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Products of the Porcine Group C Rotavirus NSP3 Gene Bind Specifically to Double-Stranded RNA and Inhibit Activation of the Interferon-Induced Protein Kinase PKR

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The porcine group C rotavirus (Cowden strain) NSP3 protein (the group C equivalent of the group A gene 7 product, formerly called NS34) shares homology with known double-stranded RNA-binding proteins, such as the interferon-induced, double-stranded RNA-dependent protein kinase PKR. A clone of NSP3, expressed both in vitro and in COS-1 cells, led to the synthesis of minor amounts of a product with an M_r of 45,000 (the expected full-length M_r of NSP3) and major amounts of products with M_rs of 38,000 and 8,000. Restriction enzyme digestion analysis prior to expression in vitro and amino-terminal sequence analysis suggest that the products with M_rs of 38,000 and 8,000 are cleavage products of the protein with an M_r of 45,000. The full-length protein and the product with an M_r of 8,000, both of which contain the motif present in double-stranded RNA-binding proteins, bound specifically to double-stranded RNA. The products with M_rs of 45,000 and 8,000 were also detected in Cowden strain-infected MA104 cells. NSP3 products expressed in COS-1 cells were capable of inhibiting activation of the double-stranded RNA-dependent protein kinase similar to other double-stranded RNA-binding proteins, and NSP3 products expressed in HeLa cells were capable of rescuing the replication of an interferon-sensitive deletion mutant of vaccinia virus.

Rotaviruses are now recognized as the major cause of severe viral gastroenteritis in humans and animals (9, 14). Rotaviruses are classified serologically into groups containing viruses that share cross-reacting antigens. Six distinct groups (A to F) of viruses have been described (1, 4, 34). Group A, B, and C rotaviruses have been found in both humans and animals; group D, E, and F rotaviruses have been found only in animals (4). Group A rotaviruses have clearly been established as causing severe diarrheal disease in young and elderly humans (19). Group B rotaviruses include viruses that have been associated with annual epidemics of severe diarrhea, primarily in human adults in China (8, 20, 28, 42, 43). Group C rotaviruses have been found in sporadic cases and outbreaks of diarrhea in piglets, cows (44), and humans (29), but the severity and number of such infections are unclear (5, 8, 39, 45).

Little is known about the functions of the rotavirus-encoded nonstructural proteins, with the exception of group A NSP4 (previously identified as NS28), which is involved in the morphogenesis of virus particles by mediating budding into the lumen of the endoplasmic reticulum (11, 23). However, most of the nonstructural proteins may be associated with viral RNA replication or with the assembly and packaging of the RNA into rotavirus cores (13, 22). In group A rotaviruses, NSP3 (the product of gene 7, formerly called NS34) is predicted to contain two major domains (3, 27): a conserved basic region that shares homology with other known single-stranded (ss) RNA-binding proteins and therefore is thought to be involved in RNA binding and a domain which contains extended regions of heptapeptide repeats of hydrophobic residues typical of alpha-helical coiled-coil structures and is likely to be involved in the formation of homooligomers or heterooligomers. Recent data suggest that the NSP3 protein of group A rotavirus has a direct role in the early stages of viral RNA metabolism and/or early assembly of replicative structures (27, 32).

The porcine group C rotavirus gene 6 has been identified as encoding the NSP3 equivalent of the group A rotaviruses (33). The porcine group C NSP3 gene encodes a protein having a predicted M_r of 45,000 as opposed to 34,600 for group A rotaviruses (2). In addition to the domains described for the group A rotaviruses, inspection of the group C NSP3 protein has identified a region at the C terminus with homology to known double-stranded (ds) RNA-binding proteins (7, 41). Such dsRNA-binding proteins include the human and mouse interferon (IFN)-induced, dsRNA-dependent protein kinase (PKR); the vaccinia virus-encoded p25 protein; *Escherichia coli* RNase III protein; the human TAR/RRE binding protein; the *Drosophila* staufen gene product; the *Saccharomyces pombe* Pac1 protein; and *Xenopus* RNA-binding protein A (41).

The vaccinia virus-encoded p25 protein and the human TAR/RRE binding protein have previously been identified as inhibitors of the PKR protein kinase (7, 31, 46). PKR is likely responsible for the IFN-induced inhibition of replication of several viruses (18, 25, 36, 38). Activation of PKR requires interaction with dsRNA leading to an autophosphorylation event. Once phosphorylated, PKR can phosphorylate exogenous substrates, including the eukaryotic protein synthesis initiation factor eIF-2. Phosphorylation of eIF-2 on its α subunit can lead to inhibition of protein synthesis by preventing the exchange of GDP for GTP on the eIF-2 complex and thereby blocking formation of the ternary complex between eIF-2, Met-tRNA, and GTP. The TAR/RRE binding protein and the vaccinia virus p25 protein inhibit PKR activation by competing with PKR for activator dsRNA (7, 31, 46).

This paper describes characterization of the porcine group C rotavirus NSP3 proteins. Expression of the NSP3 open reading frame, both in vitro and in COS-1 cells, resulted in the formation of three polypeptides having M_rs of 45,000, 38,000,

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and 8,000. Restriction enzyme digestion analysis prior to expression in vitro and amino-terminal sequence analysis suggest that the products with M_rs of 38,000 (p38) and 8,000 (p8) are cleavage products of the protein with an M_r of 45,000 (p45). p45 and p8 synthesized in vitro and in COS-1 cells bound specifically to dsRNA, while p38 appeared to have little or no affinity for dsRNA. The p45 and p8 peptides could also be immunologically detected in extracts from group C rotavirus-infected MA104 cells. NSP3 proteins synthesized in COS-1 cells were capable of inhibiting activation of the PKR protein kinase and, when expressed in HeLa cells, rescued replication of an IFN-sensitive deletion mutant of vaccinia virus.

(The nomenclature used throughout for the rotavirus nonstructural proteins is that introduced at the Fourth International Symposium of Double Stranded RNA Viruses, 12 to 16 December 1992, Scottsdale, Ariz.)

MATERIALS AND METHODS

Construction of NSP3 vectors. A cDNA clone (pC619-5) containing the entire porcine group C rotavirus (Cowden strain) NSP3 gene was obtained from Kim Green (National Institutes of Health) (33). The NSP3 gene was subcloned from pC619-5 into the eukaryotic expression vector pMT2/Va- for expression in COS-1 cells (24). Briefly, the pMT2/Va- vector (kindly provided by Randy Kaufman) was digested with *Eco*RI and *Pst*I and ligated to the pC619-5 *Eco*RI- and *Hin*dIII-digested fragment, containing the NSP3 gene, by using a *Hin*dIII-*Pst*I linker. This vector was designated pMT-NSP3.

In vitro transcription and translation. Two micrograms of pC619-5 DNA was kept circular or linearized with *Eco*RI or *Nde*I and used, according to the manufacturer's specifications, in a coupled transcription-translation system with T7 polymerase (Promega-TNT Coupled Reticulocyte Lysate Systems). For functional analysis, the translation reaction mixture contained 1 mCi of [^{35}S]methionine per ml (1,000 Ci/mmol; Dupont, NEN). For protein sequencing analysis, [^{3}H]leucine and [^{35}S]cysteine were added at 0.5 mCi/ml (200 Ci/mmol) and 0.3 mCi/ml (1,200 Ci/mmol), respectively. Coupled reaction mixtures contained 20 μM amino acid mixture minus the appropriate amino acids. The reactions were carried out at 30°C for 1 h, unless otherwise specified.

Transfection and radiolabeling of COS-1 cells. COS-1 cells were grown as monolayers and transfected as described previously (7, 15). At 48 h posttransfection, cells were labelled with 50 μCi of [^{35}S]methionine per ml (800 Ci/mmol) for 30 min and cytoplasmic extracts were prepared by detergent lysis (7, 15).

Preparation of infected cell lysates. Rhesus monkey kidney (MA104) cells were grown in tissue culture tubes (16 by 125 mm) (35). Prior to infection, confluent monolayers of MA104 cells were washed with serum-free medium and mock or Cowden infected for 1 h as previously described (22). At the indicated times postinfection, newly synthesized proteins were labeled with [^{35}S]methionine (30 μCi/ml). The infected cells were harvested, and Nonidet P-40 detergent lysates were prepared and stored at −70°C until use.

Peptide sequencing. [^{3}H]leucine- and [^{35}S]cysteine-labelled in vitro-synthesized proteins were bound to poly(rI) · poly(rC)-agarose and eluted with a spin column in Nonidet P-40 lysis buffer containing 1 M KCl (four washes of 70 μl each). The washes were pooled and diluted to a total volume of 400 μl with H_2O. Four hundred microliters of methanol and 300 μl of chloroform were added to the solution and vortexed for 3 min. Precipitated material was collected by centrifugation in a microcentrifuge (10,000 × *g*) for 30 min, after which the upper (organic) phase was carefully removed, leaving the protein precipitate which had formed between the two phases. Another 300 μl of methanol was added, and the solution was vortexed and centrifuged for 20 min. The liquid phase was discarded, and the protein pellet was dried in a Speed-Vac. After drying, the pellet was resuspended by vortexing for 5 min in 0.1% trifluoroacetic acid–10% acetonitrile (method kindly provided by Porton). The sample was loaded onto a peptide filter (Porton) and sequenced with a Porton LF3000 protein sequencer. The presence of radiolabelled amino acids in sequencing cycles was detected with a scintillation counter set with dual windows to detect ^{3}H and ^{35}S.

Poly(rI) · poly(rC)-agarose binding and competition. Poly(rI) · poly(rC)-agarose binding studies were done as previously described (26). Briefly, cytoplasmic extracts or translation mixtures were added to washed poly(rI) · poly(rC)-agarose and incubated at 4°C with continual rocking for 1 h. The agarose was washed four times in buffer A containing 150 mM KCl (10 ml per wash), and bound proteins were eluted by adding an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiling for 3 min. Proteins were separated by SDS-PAGE and detected by autoradiography.

For determining the specificity of binding, competition assays were performed as previously described (46). Prior to incubation with poly(rI) · poly(rC)-agarose, extracts were incubated with a fivefold excess (100 μg) of soluble nucleic acid for 10 min at 4°C. Nucleic acid sources included calf thymus DNA (Sigma) for ssDNA, fish sperm DNA (Sigma) for dsDNA, poly(rA) (Sigma) for ssRNA, and reovirus genome RNA or poly(rI) · poly(rC) (Pharmacia) for dsRNA.

Immunoanalysis. The entire p8 peptide was constructed with a peptide synthesizer, and the product was utilized in the production of rabbit polyclonal antiserum. The synthetic peptide injected into the rabbit was prepared as previously described (16) by cross-linking to limpet hemocyanin (Sigma) or purifying the p8 peptide by SDS-PAGE.

Western immunoblot analysis of extracts from NSP3-expressing COS-1 and MA104 cells (mock or Cowden infected) was performed as previously described (10). Briefly, extracts were first bound to poly(rI) · poly(rC)-agarose, eluted, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose, and nonspecific sites were blocked with BLOTTO. The nitrocellulose was incubated with the primary antibody and secondary goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Sigma) and developed with naphthol AS phosphate (Sigma) and fast blue BB salt (Sigma).

Immunoprecipitation of radiolabelled extracts from NSP3-expressing COS-1 cells was also performed as previously described (40). Briefly, extracts were incubated with anti-p8 serum for 1 h at 4°C and then incubated with *Staphylococcus* protein A-containing cells. The cells were washed three times in Staph A buffer (0.1 M Tris [pH 7.5], 0.15 M NaCl, 0.65% Nonidet P-40), and bound proteins were eluted and separated by SDS-PAGE. Proteins were visualized by autoradiography.

PKR kinase inhibitory activity. To assay for kinase inhibitory activity, extracts from COS-1 cells transfected with pMT-NSP3 or pMT2/Va-plasmid were added to kinase reaction mixtures containing extracts from IFN-treated mouse L cells as a source of PKR kinase activity (7, 26, 46). Kinase reaction mixtures were brought to 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–120 mM KCl–5 mM magnesium acetate–1 mM dithiothreitol–1 mM benzamidine–100 μM [γ-^{32}P]ATP (1 Ci/mmol) and indicated concentrations of reovirus dsRNA. The kinase reaction was carried out at 30°C for 15 min and was stopped by the addition

of an equal volume of 2× SDS-PAGE sample buffer and boiling for 3 min. The proteins were resolved by SDS-PAGE and detected by autoradiography.

Rescue of mutant vaccinia virus replication. The rescue of mutant vaccinia virus vP1080 replication was performed as previously described (6, 31). Briefly, HeLa cells were transfected with 20 μg of plasmid DNA as indicated or mock transfected with a $CaPO_4$-DNA coprecipitation method. The precipitate was left on the cells for 6 h and washed, and then growth media were replaced. Cells were mock infected or infected with wild-type vaccinia virus or mutant vP1080 at multiplicity of infection of 30 PFU per cell at 24 to 26 h posttransfection. At 7 h postinfection, the cells were labelled with [^{35}S]methionine (50 μCi/ml) and detergent extracts were prepared. Proteins were separated by SDS-PAGE (12% acrylamide) and visualized by autoradiography.

M_r determination. In order to obtain accurate M_rs of in vitro- and in vivo-synthesized products on SDS-PAGE, a Tricine gel system was utilized as previously described (37). A 16.5% acrylamide–3% bisacrylamide separating gel (13 cm) with a 10% acrylamide–3% bisacrylamide spacer gel (2 cm) and a 4% acrylamide–3% bisacrylamide stacking gel (1 cm) was utilized for the separation. M_rs were determined by comparison to M_r markers (M_r markers for peptides [Sigma]).

RESULTS

Identification of a consensus dsRNA-binding motif in NSP3. A conserved protein motif that is involved in dsRNA binding of the vaccinia virus E3L gene product has recently been described (7, 46) and has subsequently been identified in several other known dsRNA-binding proteins (41), including the human and mouse PKR proteins, *E. coli* and yeast RNase III, and the human-encoded TAR/RRE binding protein (Fig. 1A). Comparison of the dsRNA-binding protein consensus motif sequence (Fig. 1B) with the sequences of GenBank revealed that the porcine group C rotavirus (Cowden strain) NSP3 protein also contained a similar sequence at its C terminus (Fig. 1A and B). Comparison of the group C rotavirus NSP3 sequence with that of the simian rotavirus group A (SA-11) NSP3 equivalent revealed homology (23% identity, 41% similarity) between the first 329 amino acids of the group C protein and the full-length (315 amino acids) group A protein (Fig. 1C). However, the group C NSP3 protein contained an extra 73 amino acids on the C terminus compared with the group A protein. Given the presence of the conserved dsRNA-binding motif on the C terminus of the group C NSP3 protein, we were interested in determining if the NSP3 protein could interact with dsRNA and if this motif might be involved in binding.

Analysis in vitro. NSP3 gene products were synthesized from plasmid pC619-5 by using a coupled transcription-translation system. As shown in Fig. 2A, three major translation products were detected, having approximate M_rs of 45,000, 38,000, and 8,000 (lane D). A fourth product, with an approximate M_r of 28,000, was observed but was also detected in transcription-translation reaction mixtures lacking exogenously added DNA (Fig. 2A, compare lanes D and E). Synthesis of the products with M_rs of 45,000 (p45), 38,000 (p38), and 8,000 (p8) was dependent on the presence of the pC619-5 plasmid.

The in vitro-synthesized proteins were assayed for the ability to bind to dsRNA. As shown in Fig. 2A, the p45 and p8 proteins bound to a dsRNA-agarose resin but did not bind to an agarose matrix (compare lanes B and A). Similar results were observed with both poly(rI) · poly(rC)-agarose and reovirus dsRNA-agarose (data not shown). The in vitro-synthesized p38 protein did not interact with the dsRNA-agarose resin (Fig. 2A, lane B).

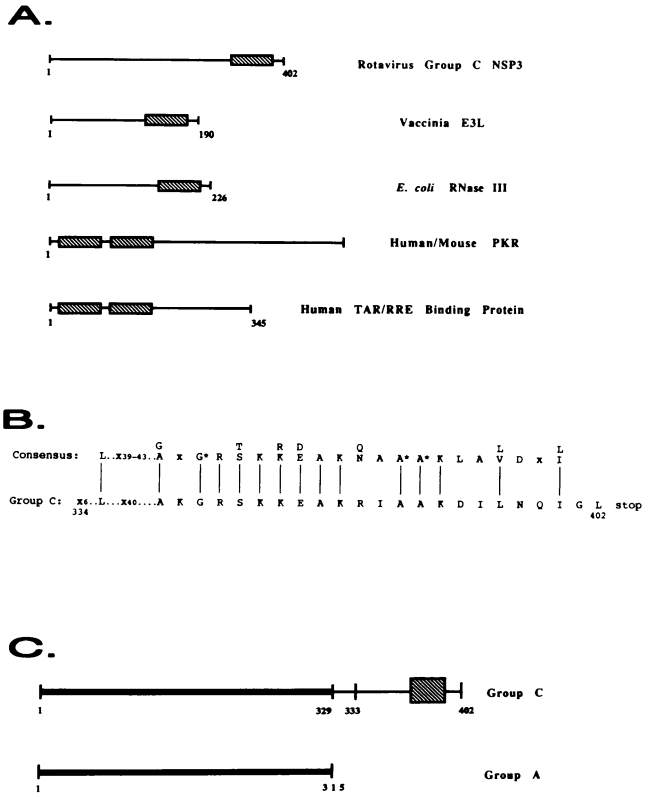

FIG. 1. Regions of sequence homology. (A) The amino acid sequences of several known dsRNA-binding proteins were compared, and regions of homology are illustrated as hatched boxes. Hatched box regions include group C rotavirus NSP3 (amino acids 380 to 400), vaccinia virus E3L gene product (amino acids 162 to 182), *E. coli* RNase III (amino acids 203 to 223), human PKR (amino acids 54 to 74 and 145 to 165), mouse PKR (amino acids 53 to 73 and 139 to 159), and human TAR/RRE binding protein (amino acids 53 to 73 and 183 to 203). (B) From the known dsRNA-binding proteins described for panel A, a consensus dsRNA-binding sequence was constructed. Asterisks denote amino acids which have been shown to be required for dsRNA binding as determined by mutational analysis of the vaccinia virus E3L gene product. Alignment of the group C rotavirus NSP3 p8 product with the consensus sequence is shown. (C) The amino acid sequences of porcine group C rotavirus NSP3 and the simian (SA-11) rotavirus group A NSP3 were compared. Darkened regions represent overlapping sequence homology and/or similarity between the two proteins (from amino acids 1 to 329 for group C and full-length group A). The hatched box represents the conserved dsRNA-binding motif. Amino acid 333 is marked as the proteolytic cleavage site of group C NSP3.

To determine the specificity of this dsRNA interaction, competition experiments were performed. Incubation with a fivefold excess of dsRNA [poly(rI) · poly(rC) or reovirus dsRNA] eliminated p8 binding to the matrix (Fig. 2B, lane C). Preincubation with a fivefold excess of dsDNA, ssRNA, or ssDNA did not reduce the amount of p8 binding to the dsRNA-linked matrix (Fig. 2B, lanes A, B, and D). This suggests that p8 specifically bound to dsRNA. Incubation with dsRNA eliminated p45 binding to the dsRNA-agarose matrix (Fig. 2B, lane C). Decreased levels of p45 binding were often observed in competition assays with ssDNA. However, the

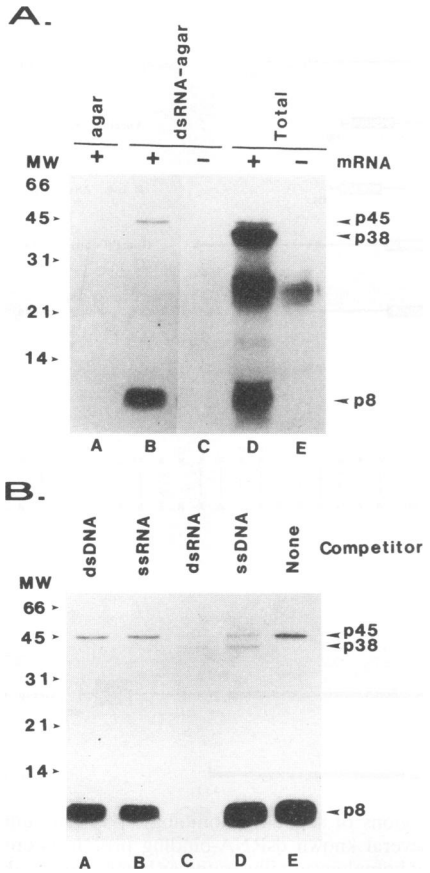

FIG. 2. In vitro synthesis of NSP3 gene products. (A) The NSP3 gene was transcribed and translated in vitro by using plasmid pC619-5 in the presence of [^{35}S]methionine. Products formed were separated by SDS-PAGE and detected by autoradiography. Coupled transcription-translation reactions were performed either in the presence (lanes A, B, and D) or absence (lanes C and E) of plasmid DNA. Lanes D and E represent the total translation products (2 μl). The remaining lanes illustrate products (50 μl of translation reaction) which bound to dsRNA-agarose (lanes B and C) or Sepharose CL-6B alone (lane A). (B) In vitro-radiolabelled transcription-translation products of the NSP3 gene were preincubated with 100 μg of dsDNA (lane A), ssRNA (lane B), dsRNA (lane C), or ssDNA (lane D) or no nucleic acid (lane E), followed by incubation with dsRNA-agarose (containing 20 μg of dsRNA). The proteins bound to the resin were eluted and separated by SDS-PAGE and detected by autoradiography.

level of this competition varied between translation reactions, and binding was never reduced more than twofold. Interestingly, incubation with ssDNA caused a small amount of p38 to be bound to the dsRNA-agarose matrix (approximately 0.01%) compared with the amounts of p8 and p45 bound (16 and 0.6%, respectively).

In order to determine the nature of the three products encoded by the NSP3 gene, further analysis was performed. The electrophoretic mobility of the p45 product is in good agreement with the predicted M_r encoded by the long open reading frame of the NSP3 gene (402 amino acids, predicted M_r of 45,125) (33). No other AUG codons are present within the first 118 codons of the NSP3 open reading frame, suggesting that p38 synthesis is not the result of an internal initiation event. The M_rs of p8 and p38 are consistent with their being proteolytic cleavage products of the full-length p45 protein. A unique restriction site, *Nde*I, was present on the NSP3 gene immediately upstream of the putative dsRNA-binding motif (Fig. 3A). In vitro translation of *Nde*I-digested DNA would result in the synthesis of a product of 365 amino acids. As shown in Fig. 3A, in vitro transcription and translation of *Nde*I-digested DNA resulted in the synthesis of only one major product with an M_r of 38,000 that would coincide with the predicted M_r of the full-length product (lane D). The p38 NSP3 protein was not detected at a lower M_r, as would have been expected had *Nde*I cleavage significantly altered the C terminus of this protein. Therefore, these results suggest that the p38 NSP3 coding region does not overlap the *Nde*I site and therefore does not contain the C-terminal region of the full-length protein. Given the M_r of the p38 protein and the lack of internal protein synthesis initiation sites, p38 presumably contains the entire N-terminal coding region of the NSP3 protein. The synthesis of p8 was not observed upon transcription and translation of *Nde*I-digested DNA (Fig. 3A, lane D). This suggests that p8 is encoded downstream of the *Nde*I site at the C terminus of the NSP3 protein.

In order to determine the N terminus of the p8 gene product, protein sequencing of p8 that had been labelled with radioactive leucine and cysteine was performed. The results indicate that p8 contains a leucine at residues 4 and 7 and a cysteine at residue 10 (Fig. 3B). Alignment with the entire NSP3 sequence indicates that p8 begins with an N-terminal proline coinciding with amino acid 334 of the full-length NSP3 protein (Fig. 3C). Since there are no methionines encoded within 90 codons upstream of this position, it is unlikely that p8 could have been formed through an internal initiation event and therefore was presumably formed through proteolysis. p38 and p8 were present in molar ratios of 1:1.4, while p45 was present in much smaller amounts, again consistent with the occurrence of a proteolytic cleavage event. A more precise determination of the M_r of p8 with a Tricine gel system suggests an M_r of 7,900 (data not shown). The predicted M_r of the protein formed by proteolytic cleavage at proline 334 to the C terminus of NSP3 would be 7,864. Encoded within this region is the consensus putative dsRNA-binding motif.

Cleavage of p45 to p38 and p8 appears to occur either cotranslationally or very rapidly posttranslationally. A coupled transcription-translation reaction, using pC619-5, was performed for various lengths of time and assayed for the rate of synthesis of p45, p38, and p8. As shown in Fig. 4, p38 and p8 were detected 20 min after the start of the reaction, 5 min prior to the detection of p45. This suggests that proteolysis occurred cotranslationally with the synthesis of p45 or immediately after the synthesis of p45.

Analysis in a eukaryotic expression system and infected cells. In order to characterize NSP3 synthesis and function within a cell, the NSP3 gene was subcloned into the eukaryotic expression vector pMT2/Va-. Transfection of COS-1 cells with this plasmid, pMT-NSP3, led to the expression and synthesis of p45, p38, and p8 (Fig. 5A). Only the synthesis of p38 could be detected in total cytoplasmic extracts separated on an SDS-PAGE gel (Fig. 5A, lane A), presumably because of the low levels of p45 as a result of proteolytic cleavage and the small number of methionines (two) present in p8. However, the presence of p45 and p8 in these extracts could be detected after concentration by binding to dsRNA-agarose (Fig. 5A, lane D). In relation to p8 and p45, much lower levels of p38 bound to the dsRNA-agarose. Three endogenous cellular proteins with M_rs of >80,000 also bound to the dsRNA-agarose resin (Fig. 5A, lanes D, E, and F, and 5B). The nature and identity of these proteins are unknown.

To determine the specificity of dsRNA interaction of the in

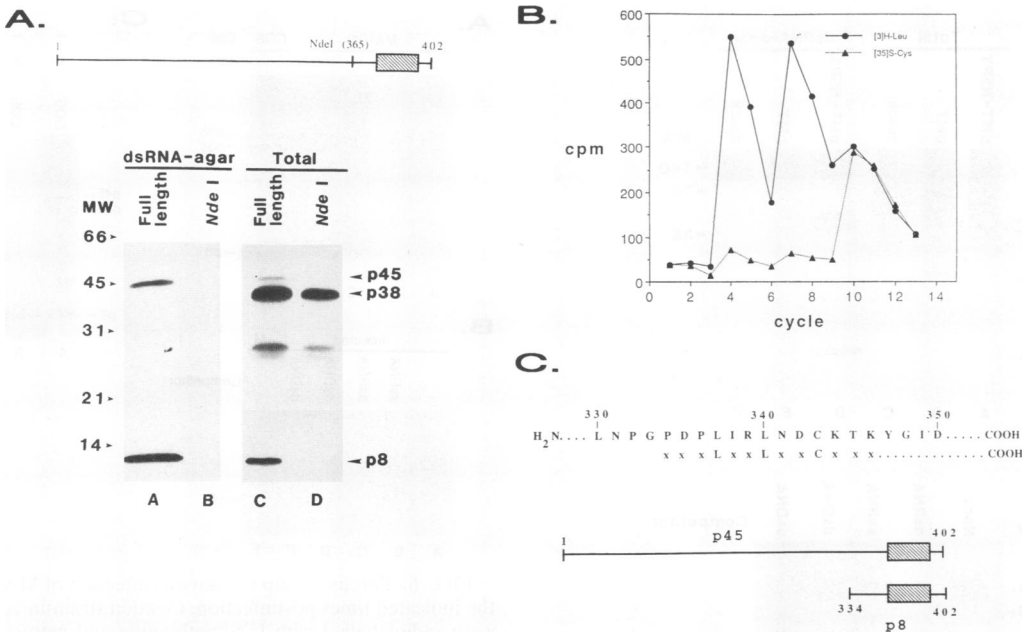

FIG. 3. Mapping of the NSP3 proteolytic cleavage products. (A) The pC619-5 plasmid was linearized with *Eco*RI (lanes A and C) or *Nde*I (lanes B and D), followed by coupled in vitro transcription and translation in the presence of [^{35}S]methionine. Products were separated by SDS-PAGE and visualized by autoradiography. Lanes C and D represent total translation products (0.5 μl). Lanes A and B represent translation products (50 μl) which were bound to dsRNA-agarose. (B) In vitro-synthesized NSP3 gene products were labelled with [^{3}H]leucine and [^{35}S]cysteine, followed by purification on dsRNA-agarose and elution. Sequence analysis of the eluted protein was performed and each cycle was assayed for ^{3}H and ^{35}S. The graph represents counts per minute obtained for each cycle with each corresponding detection window. (C) Alignment of the NSP3 amino acid sequence with the radiolabelled cycles ([^{3}H]leucine and [^{35}S]cysteine) obtained from sequence analysis. Hatched boxes represent the conserved dsRNA-binding motif.

vivo-synthesized NSP3 gene products, competition assays were performed as previously described (Fig. 2B). p45, p38, and p8 interaction with the dsRNA was specific since binding to dsRNA-agarose could only be inhibited with excess soluble dsRNA and not with excess soluble ssRNA, ssDNA, or dsDNA (Fig. 5B).

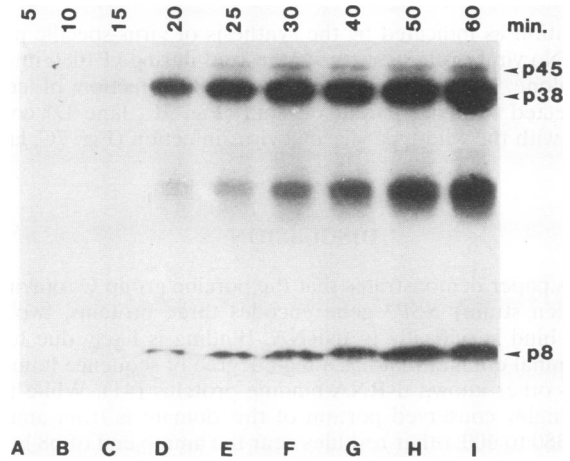

FIG. 4. Immediate proteolytic processing of p45. Coupled in vitro transcription-translation reactions were performed with pC619-5 in the presence of [^{35}S]methionine. Reactions were stopped at indicated times by the addition of 2× SDS-PAGE sample buffer and boiling. Translation products were separated by SDS-PAGE and visualized by autoradiography.

The synthesis of a protein with properties similar to p8 was also detected in MA104 cells infected with porcine group C rotavirus. Synthesis of a dsRNA-binding protein that comigrated with the COS-1-synthesized p8 was detectable by 3 h postinfection (Fig. 6A, lanes B and E). This product was not observed in uninfected cells (Fig. 6A, lane A). Binding of this virus-induced polypeptide to dsRNA was specific since binding could be inhibited with excess soluble dsRNA but could not be inhibited with excess soluble ssRNA, ssDNA, or dsDNA (Fig. 6B). Western blot analysis with antiserum developed against a synthetic p8 peptide demonstrated the presence of both p8 and p45 in NSP3-expressing COS-1 cells and in group C rotavirus-infected MA104 cells (Fig. 6C, lanes B and D). These proteins could not be detected in mock-infected MA104 cells (Fig. 6C, lane C). These results suggest that p8 synthesis occurred during the group C rotavirus infection.

The presence of p38 could not be detected by Western blot analysis with anti-p8 antiserum either in NSP3-expressing COS-1 cells or in the Cowden-infected MA104 cells (Fig. 6C, lanes B and D). This result is in agreement with p45 processing resulting in the formation of p38 and p8. However, anti-p8 immunoprecipitation of radiolabelled extracts from NSP3-expressing COS-1 cells resulted in the detection of p45, p8, and p38 (Fig. 6C, lane A). These results suggest that p38 was coprecipitated in a complex with either p8 or p45.

Functional analysis. Several dsRNA-binding proteins have been shown to function as inhibitors of the IFN-induced eIF-2α protein kinase PKR. To determine if NSP3 proteins could also act as inhibitors of PKR, kinase assays, measuring the level of PKR autophosphorylation, were performed in the presence and absence of COS-1 cell extracts containing NSP3.

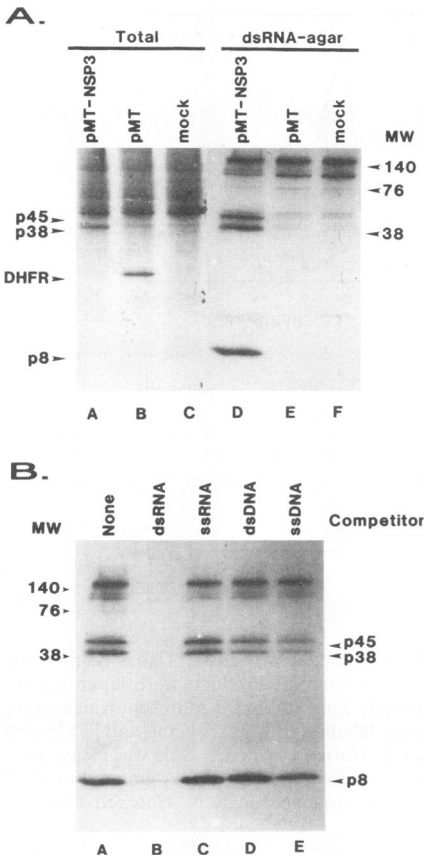

FIG. 5. In vivo synthesis of NSP3 gene products. (A) COS-1 cells were left untransfected (lanes C and F) or transfected with pMT (lanes B and E) or pMT-NSP3 (lanes A and D) and radiolabelled with [^{35}S]methionine 48 h posttransfection. Radiolabelled products were separated by SDS-PAGE and visualized by autoradiography. Lanes A to C represent total cytoplasmic extracts (5 μl). Lanes D to F represent extracts bound to dsRNA-agarose (100 μl). DHFR, dihydrofolate reductase encoded by pMT. (B) Extracts from radiolabelled COS-1 cells transfected with pMT-NSP3 were preincubated with no nucleic acid (lane A) or 100 μg of dsRNA (lane B), ssRNA (lane C), dsDNA (lane D), or ssDNA (lane E), followed by incubation with dsRNA-agarose (containing 20 μg of dsRNA). The proteins bound to the resin were eluted and separated by SDS-PAGE and detected by autoradiography.

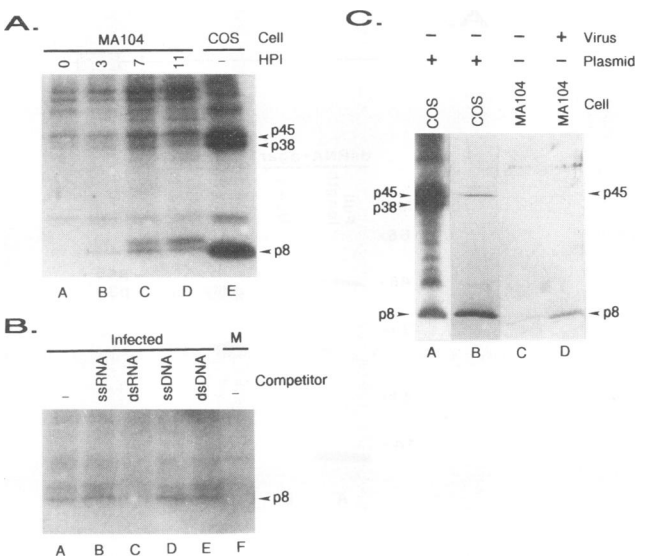

FIG. 6. Porcine group C rotavirus infection of MA104 cells. (A) At the indicated times postinfection, Cowden strain-infected MA104 cells were radiolabelled with [^{35}S]methionine and extracts were prepared (lanes B to D). Radiolabelled extracts from mock-infected MA104 cells (lane A) and from COS-1 cells expressing NSP3 (lane E) were also prepared. Extracts were incubated with dsRNA-agarose, and bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. (B) Group C rotavirus-infected MA104 cells were radiolabelled with [^{35}S]methionine at 11 h postinfection, and extracts were prepared. These extracts were preincubated with no nucleic acid (lane A) or 100 μg of ssRNA (lane B), dsRNA (lane C), ssDNA (lane D), or dsDNA (lane E), followed by incubation with dsRNA-agarose (containing 20 μg of dsRNA). The proteins bound to the resin were eluted and separated by SDS-PAGE and detected by autoradiography. (C) Extracts were prepared from group C rotavirus-infected (lane D) or mock-infected (lane C) MA104 cells (11 HPI) or from COS-1 cells expressing NSP3 (lane B). These extracts were incubated with dsRNA-agarose, and bound proteins were eluted, separated by SDS-PAGE, and analyzed by Western blot analysis with anti-p8 serum. Lane A represents an immunoprecipitation of a ^{35}S-labelled cell extract from NSP3-expressing COS-1 cells by using anti-p8 serum.

As shown in Fig. 7A, in the absence of NSP3, PKR autophosphorylation required the presence of approximately 0.03 μg of dsRNA per ml. However, when extracts containing NSP3 products were added to the kinase assay, a 10-fold higher concentration of dsRNA was required to achieve similar levels of PKR autophosphorylation (compare Fig. 7A, lane C, and 7B, lane E). These results suggest that NSP3 is capable of inhibiting PKR activation.

A vaccinia virus mutant (vP1080), with the E3L gene which encodes the dsRNA-binding protein p25 deleted, has recently been characterized (6). p25 can function both in vivo and in vitro as a PKR inhibitor (7, 46). The replication of vP1080 is blocked in HeLa cells. Replication of vP1080 can be rescued by transfection of any of several genes encoding dsRNA-binding protein inhibitors of PKR into cells (6, 31). We therefore assayed the ability of the NSP3 gene product or products to rescue vP1080 replication. As shown in Fig. 7C, both p25 and NSP3 (lanes F and E, respectively) were able to rescue vP1080 replication as indicated by the synthesis of virus-specific proteins. No viral proteins were synthesized during vP1080 infection alone (Fig. 7C, lane C) or vP1080 infection of cells transfected with the parent plasmid (Fig. 7C, lane D) compared with the wild-type vaccinia virus infection (Fig. 7C, lane B).

DISCUSSION

This paper demonstrates that the porcine group C rotavirus (Cowden strain) NSP3 gene encodes three proteins, two of which bind specifically to dsRNA. Binding is likely due to a C-terminal domain that has a high degree of sequence homology to other known dsRNA-binding proteins (41). While the most highly conserved portion of this domain is from amino acids 380 to 400, other residues near the amino end of p8 have been conserved. Leucine 340 is conserved in nearly all proteins containing this motif (41), suggesting that nearly the entire p8 sequence may be included in the dsRNA-binding motif. As observed in this paper, the p8 peptide specifically binds to dsRNA, along with the full-length NSP3 gene product p45, both of which contain the conserved dsRNA-binding motif. To

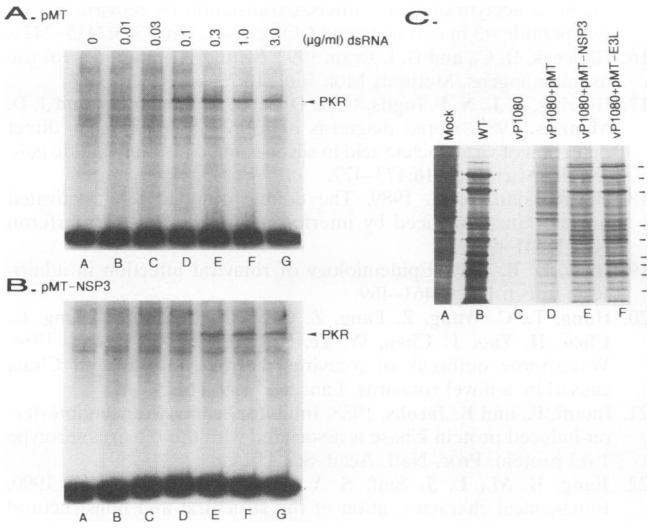

FIG. 7. Physiological activities associated with NSP3. (A and B) PKR kinase autophosphorylation was performed in the presence of [γ-^{32}P]ATP and increasing concentrations of dsRNA. (A) Incorporation of radioactivity into PKR in the presence of exogenously added extracts from pMT-transfected COS-1 cells. (B) Incorporation of radioactivity into kinase in the presence of exogenously added extracts from pMT-NSP3-transfected COS-1 cells. (C) Rescue of vP1080 replication. HeLa cells were transfected with the indicated plasmid (pMT, lane D; pMT-NSP3, lane E; pMT-E3L, lane F; no plasmid, lanes A to C), followed by mock infection (lane A), vaccinia virus infection (lane B), or vP1080 infection (lanes C to F). Radiolabelled extracts were prepared, and proteins were separated by SDS-PAGE and visualized by autoradiography.

date, p8 appears to be the smallest natural peptide described which is capable of specific dsRNA binding.

Other dsRNA-binding proteins have been shown to be inhibitors of the IFN-induced protein kinase PKR. Such proteins include the vaccinia virus p25 protein, the reovirus σ3 protein, and the human TAR/RRE binding protein (7, 21, 31, 46). With the virus-encoded p25 and σ3 proteins, this inhibition has been suggested to be involved in IFN resistance observed during both viral infections (7, 21, 46). The IFN-induced PKR requires dsRNA for activation leading to an inhibition in the initiation of translation (18). Inhibition of PKR by virus-encoded dsRNA-binding proteins appears to occur by saturation of binding sites on dsRNA molecules, thereby blocking PKR association (7, 21, 31, 46). The group C rotavirus NSP3 gene products are also capable of inhibiting the activation of PKR. While interaction of p45 or p38 could be responsible for this activity, the molar amount of p8 bound to dsRNA far exceeded the molar amount of p45 bound, suggesting that p8 is likely responsible for the kinase inhibitory activity. Furthermore, the NSP3 products are capable of rescuing the replication of an IFN-sensitive vaccinia virus mutant with the dsRNA-binding protein p25 deleted. Again, p8 is likely involved in this activity, since viral rescue probably requires the presence of a dsRNA-binding protein. Physiologically, inhibition of PKR activity and the viral rescue assay suggest that the group C rotaviruses could be resistant to IFN, as observed with other viruses encoding similar dsRNA-binding proteins.

The porcine group C rotavirus (Cowden strain) NSP3 gene encodes a full-length polypeptide with an M_r of 45,000 (p45) which is subsequently proteolytically cleaved into polypeptides with M_rs of 38,000 (p38) and 8,000 (p8). This proteolytic event occurred very rapidly after the synthesis of p45 or may have occurred concomitantly during the synthesis of p45. Since high concentrations of proteolytic enzymes should not be and have not been detected in the in vitro system and since identical proteolytic events are observed in vivo, in both infected cells (Fig. 6) and transfected cells (Fig. 5), these results may suggest an autoproteolytic event. Recently, a similar proteolytic cleavage site has been described for the cardioviral P2 region (30). Primary 2A/2B cleavage occurs cotranslationally at the consensus amino acid cleavage motif of D-V-E-X-N-P-G-P, with cleavage occurring between the G and the final P. The porcine group C rotavirus NSP3 protein contains the sequence 327-D–V–E–L–N–P–G–P-334 at its cleavage site. NSP3 cleavage occurs between G-333 and P-334, resulting in the formation of p38 and p8. Preliminary evidence in the cardiovirus system suggests that this motif may lead to spontaneous hydrolysis of the G-P peptide bond (30).

The NSP3 genes of rotavirus group A strains have previously been shown to encode a product with an M_r of 34,600 (p34) (2). Alignment of the amino acid sequence of group A (SA-11 strain) genome segment 7 (which encodes the NSP3 protein) with the group C equivalent shows a high degree of similarity between the entire group A sequence and the first 329 amino acids of the group C NSP3 protein. Proteolytic cleavage of the group C NSP3 protein occurred between amino acids 333 and 334, leading to the production of p38 at the N terminus and p8 at the C terminus. Since the group A p34 protein has significant sequence similarity with p38, p38 would likely have physiological activities similar to those of the p34 protein. Therefore, the results presented in this paper suggest that the group C NSP3 protein p45 has an extra 69-amino-acid sequence placed at the C terminus of a group A p34-like polypeptide, p38. As previously described and in agreement with the results in this paper, the separation of group A rotavirus genomic segments by SDS-PAGE always gives a characteristic electropherotype of 4-2-3-2, whereas group C rotaviruses have an electropherotype of 4-3-2-2 (17). The genomic segment responsible for the shifted electropherotypic pattern is the genome segment encoding NSP3. This suggests the presence of a larger open reading frame in all group C isolates as yet characterized. Recently, the group B NSP3 equivalent has been identified (12). The group B NSP3 open reading frame is also larger than that identified for the group A NSP3; however, no significant sequence homology to the consensus dsRNA-binding motif has been identified.

The group A NSP3 protein appears to consist of two major domains (3, 27). The first is a basic region conserved in group A and group C that is thought to be involved in ssRNA binding. This nucleic acid interaction is thought to be involved in genome replication and/or viral assembly. The second domain of group A NSP3 contains extended regions of heptapeptide repeats of hydrophobic residues. This region has been suggested to participate in forming coiled-coil oligomers. Oligomers of NSP3 have been isolated, including homodimers and heterooligomers associated with NSP1 (previously identified as NS53). As described in this paper, minor amounts of the group C p38 proteolytic product (group A NSP3 equivalent) expressed in vivo were detected as interacting with dsRNA-agarose. Presumably, this interaction is due to either RNA binding through the p38 basic domain or heterodimerization of p38 with p45 and subsequent binding of p45 to the dsRNA matrix. This second case is suggested, since equimolar amounts of p38 and p45 were detected among the bound proteins. Furthermore, gel filtration analysis of extracts from COS-1 cells transfected with pMT-NSP3 suggested that no detectable

p38 was monomeric, but instead p38 was eluted in the void volume of the column (M_r of >200,000) where a significant portion of p45 was also observed to elute (unpublished observations). Finally, p8-specific antiserum recognized only p8 and p45 by Western blot analysis; however, it could immunoprecipitate p8, p45, and p38 (Fig. 6). This result suggests that p38 was complexed with either p45 or p8 and was coprecipitated with the antiserum. Therefore, the p38 protein observed in dsRNA-agarose affinity assays (Fig. 5) was likely the result of oligomerization with p45. It should be noted that normally we could not detect oligomerization when proteins were synthesized in vitro, presumably because of the presence of reducing agent in the reticulocyte lysate (20).

The results presented in this paper suggest that the group C rotavirus NSP3 protein may have multiple physiological functions involved in viral replication. The full-length NSP3 protein is subsequently cleaved into p38 and p8. The p38 polypeptide is likely involved in genomic replicase and assembly activity, as has been suggested for the group A NSP3 protein (27). However, a novel activity associated with the group C NSP3 protein is suggested. The p8 proteolytic cleavage product, because of its known association with dsRNA, may be involved in regulation of the IFN-induced protein kinase PKR.

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