

Role for Bovine Viral Diarrhea Virus E^{rns} Glycoprotein in the Control of Activation of Beta Interferon by Double-Stranded RNA

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Production of alpha/beta interferon in response to viral double-stranded RNA (dsRNA) produced during viral replication is a first line of defense against viral infections. Here we demonstrate that the E^{rns} glycoprotein of the pestivirus bovine viral diarrhea virus can act as an inhibitor of dsRNA-induced responses of cells. This effect is seen whether E^{rns} is constitutively expressed in cells or exogenously added to the culture medium. The E^{rns} effect is specific to dsRNA since activation of NF- κ B in cells infected with Semliki Forest virus or treated with tumor necrosis factor alpha was not affected. We also show that E^{rns} contains a dsRNA-binding activity, and its RNase is active against dsRNA at a low pH. Both the dsRNA binding and RNase activities are required for the inhibition of dsRNA signaling, and we discuss here a model to account for these observations.

Bovine viral diarrhea virus (BVDV) is a *Pestivirus* belonging to the family *Flaviviridae* and is a major pathogen of cattle throughout the world with an incidence of infection often in excess of 70% (9, 24). An unusual feature of pestiviruses is their ability to sustain a persistent infection after in utero infection of a fetus. BVDV exists as two biotypes according to their cytopathogenicity on cultured cells, and the ability to sustain a persistent infection is exclusively a property of the noncytopathogenic BVDV biotype. Persistently infected calves born after infection of the fetus during the early stages of pregnancy are held to be the source of acute virus infection and the subsequent generation of cytopathogenic BVDV variants isolated from animals with mucosal disease. Survival of the virus in the fetus requires avoiding immune responses; since the acquired immune response is not developed early in embryogenesis, innate immune responses are the main host defenses from infection. Evasion of innate immunity in the fetus is at least in part due to the lack of alpha/beta interferon (IFN- α/β) production after noncytopathogenic BVDV infection (6). We and others have shown that cultured cells infected with noncytopathogenic BVDV are refractory to the IFN-inducing effects of infection with heterologous virus or the addition of double-stranded RNA (dsRNA) to cells (3, 30). Thus, noncytopathogenic BVDV takes active measures to block the production of IFN- α/β . A key component of this inhibition operates at the level of inhibiting the DNA-binding activity of the IFN regulatory transcription factor IRF-3 induced by heterologous virus infection (2).

The viral gene products associated with the evasion of the IFN system have not been identified. The virus genome is positive-strand RNA that is translated to form a single virus polyprotein, which, through cleavage by both host and virus proteases, gives rise to either 11 or 12 mature viral proteins (NH2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH), depen-

dent on the virus biotype (20). E^{rns}, E1, and E2 are glycoproteins found on the surface of the virion (35). In addition to being a virion protein, a proportion of E^{rns} is secreted from infected cells into the extracellular environment as a consequence of it lacking a typical transmembrane anchor domain (28). The biological significance of secreted E^{rns} has not been determined. However, E^{rns} has two defined activities. First, E^{rns} has an RNase activity (10, 29, 37) that shows a strong preference for single-stranded RNA (ssRNA) (37). Second, E^{rns} glycoproteins of both BVDV and a closely related pestivirus, classical swine fever virus (CSFV), have been shown to bind to cell surface glycosylaminoglycans (13, 14) through residues located close to their C termini (12, 15). The effects of inactivating the RNase activity on viral function are ambiguous: it has been reported that a genetically engineered strain of CSFV that lacks RNase activity induced cell death in swine kidney cells, but the parent virus did not (11); however, another mutant CSFV lacking RNase activity was not cytopathogenic (23).

In the present study we have investigated the properties of secreted BVDV E^{rns}, and we show that this protein can bind to dsRNA-agarose and inactivate dsRNA-dependent signaling events initiated by the presence of dsRNA in the medium. Through the construction of mutated E^{rns} by expression of plasmids subjected to site-directed mutagenesis at sites within the E^{rns}-coding sequence (see Fig. 1), we show that this inhibition required the RNase activity and a region that influenced E^{rns} binding to dsRNA.

MATERIALS AND METHODS

Cell culture and generation of cell lines. Calf testes (CTe) cells were used between passages 4 and 7 and were cultured in Dulbecco modified Eagle medium (DMEM), supplemented with penicillin and streptomycin, and 10% fetal calf serum (FCS) free of both BVDV and anti-BVDV antibody. Human MG-63 cells were grown in DMEM supplemented with 10% FCS and penicillin and streptomycin. MDBK cells were cultured in Eagle minimal essential medium supplemented with 1% nonessential amino acids and 10% FCS, and insect (Schneider *Drosophila* S2) cells were obtained from Invitrogen and cultured at room temperature in Schneider's *Drosophila* medium (also from Invitrogen) containing 10% FCS.

The full-length E^{rns} gene from noncytopathogenic BVDV strain pe515, preceded at the 5' end by the CD33 signal peptide sequence in order to ensure

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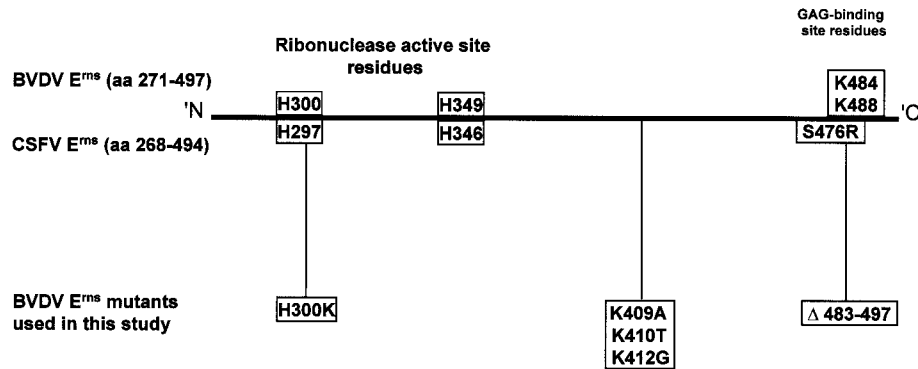


FIG. 1. Schematic representation of the primary amino acid sequence of the BVDV and CSFV glycoprotein E^{rn}s. Residues critical for RNase activity and for glycosaminoglycan binding (11, 12, 15, 23) and the mutants used in the present study are indicated.

efficient E^{rn}s secretion, was amplified from the previously cloned Signal/pIg/Fc plasmid (14) by using PCR, and cloned between the *Nhe*I and *Xba*I sites of the mammalian expression vector pcDNA6/V5-His (Invitrogen) to generate pcDNA6/CD33-E^{rn}s/V5-His. The forward primer used was 5'-ACCCAAGCTGCTAGCTCAGACATGCCG-3', which corresponds to nucleotides 6267 to 6293 of the Signal/pIg/Fc plasmid (R&D Systems), incorporating an *Nhe*I restriction enzyme recognition site at the 5' end of the region encoding the CD33 signal peptide. The reverse primer (pIgplus SEQ 3' R&D Systems) sequence was 5'-ATGTGTGAGTTTGTCAACAAG-3', which corresponds to nucleotides 101 to 121 of the Signal/pIg/Fc plasmid. An equivalent plasmid expressing a CD33-tagged BVDV E2 protein (pcDNA6/CD33-E2/V5-His) was generated in a similar manner. Stably transformed MDBK cell lines were established for constitutive transgene expression of E^{rn}s, E2, or β -galactosidase by transfection with pcDNA6/CD33-E^{rn}s/V5-His, pcDNA6/CD33-E2/V5-His, or pcDNA6/lacZ/V5-His (Invitrogen), respectively, by using FuGENE 6 (Roche Biochemicals) according to the manufacturer's instructions. The cells were treated with blasticidin (10 μ g/ml), and pools of blasticidin-resistant cells were selected.

Construction of mutants of E^{rn}s. The E^{rn}s gene was cloned previously into a *Drosophila melanogaster* expression vector to produce pMT/BiP/E^{rn}s/V5-His, which represented the full-length E^{rn}s cDNA of noncytopathogenic BVDV strain pe515 corresponding to amino acids 271 to 497 of the BVDV polyprotein (14). The construction of mutant E^{rn}s Δ 483-497 lacking amino acid residues 483 to 497 in the carboxy terminus of E^{rn}s and the construction of the mutants E^{rn}sK409A, E^{rn}sK410T, E^{rn}sK412G, and E^{rn}sK409A-K410T, containing specific substitutions of lysine residues for uncharged amino acids at positions 409, 410, and 412, respectively, in the E^{rn}s polypeptide, have been described previously (15). The schematic location of these E^{rn}s mutants is shown in Fig. 1. The RNase-negative mutant, E^{rn}sH300K, was constructed from pMT/BiP/E^{rn}s/V5-His by PCR-mediated mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's guidelines. The resultant plasmid [pMT/BiP/E^{rn}s(H300K)/V5-His] was cotransfected with a hygromycin-resistant selection vector pCoHYGRO (Invitrogen) at a ratio of 19 [pMT/BiP/E^{rn}s(H300K)/V5-His] to 1 (pCoHYGRO) into insect (S2) cells by using a calcium phosphate transfection kit (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected with hygromycin B (Invitrogen), and mutant E^{rn}s-expressing cell lines were established from clones selected by limiting dilution.

Expression and purification of E^{rn}s expressed in insect cells. Wild-type and mutant E^{rn}s glycoproteins were expressed as described previously (14). Briefly, *Drosophila* S2 cells harboring the E^{rn}s gene were cultured in 150 ml of Schneider's *Drosophila* medium containing 10% FCS. After 5 to 6 days at room temperature the medium was replaced with 500 ml of *Drosophila* medium free of serum (Invitrogen) containing L-glutamine (90 mM), and expression of E^{rn}s was induced by the addition of 7.5 μ M copper sulfate. At 4 to 5 days postinduction, the cells were harvested, and the culture supernatant was applied directly to a 10-ml chelating-Sepharose column (Amersham Biotech), which had previously been equilibrated with 20 mM phosphate buffer (pH 7.8) containing 300 mM NaCl. The column was washed with 20 mM phosphate buffer (pH 6.0) containing 1.0 M NaCl and 100 mM imidazole, and E^{rn}s was eluted with 20 mM phosphate buffer (pH 6.0) containing 1.0 M NaCl and 400 mM imidazole. Fractions containing E^{rn}s were pooled and dialyzed against phosphate-buffered saline (PBS). The purity of the glycoproteins was assessed by using sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-10% PAGE), and the concentration of the purified proteins was determined by using the Coomassie Plus-200 protein assay reagent (Pierce). The stability of the wild type and mutant E^{rn}s was assessed after the addition of the purified glycoproteins to cell culture maintenance medium and incubation at 37°C for 18 h, followed by PAGE.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were carried out to analyze the inhibition of NF- κ B activation by E^{rn}s. Confluent CTE and MG-63 cells were cultured in 9-cm dishes, and the cells were either mock treated or treated with polyribinosinic acid-polyribocytidylic acid [poly(rI)-poly(rC); 5 μ g/ml], human tumor necrosis factor alpha (TNF- α ; 10 ng/ml), or infected with Semliki Forest virus (SFV) at a multiplicity of infection of \sim 5 PFU/cell in the presence or absence of E^{rn}s (1 μ g/ml). Nuclear extracts were prepared as previously described (36), the protein concentrations were determined by a Bradford assay (Bio-Rad), and 10- μ g aliquots of extracts were assayed by using the PRD II element from the human IFN- β promoter as a probe for NF- κ B, as described by Visvanathan and Goodbourn (31).

Immunoblot analysis. Induction of MxA protein by dsRNA was determined by immunoblot analysis. Confluent monolayers of CTE cells cultured in 24-well plates were either mock treated, treated with in vitro-transcribed dsRNA (5 μ g/ml), or treated with poly(rI)-poly(rC) (5 μ g/ml) and E^{rn}s (1 μ g/ml). After 18 h at 37°C, the cells were washed once with PBS and treated with 100 μ l of SDS-PAGE loading buffer (8 M urea, 10 mM Tris-HCl [pH 6.8], 2% SDS, 2% 2-mercaptoethanol, 0.1% bromophenol blue). The samples were boiled for 2 min, a 10- μ l portion of the cell lysates was subjected to SDS-PAGE, and the separated proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biotech). After the membrane was blocked with 5% (wt/vol) skimmed milk in PBS containing 0.1% Tween 20 (PBS-T), the membrane was probed for MxA protein by using a rabbit antiserum raised against human MxA (serum no. 41; a generous gift from P. Staeheli, Freiburg, Germany) at a dilution of 1:2,000 and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma) at a dilution of 1:5,000. The bound antibody was visualized by using an ECL detection kit (Amersham Biotech) according to the manufacturer's instructions.

IFN-specific RT-PCR. Confluent monolayers of CTE cells cultured in 24-well plates were either mock treated or treated with poly(rI)-poly(rC) (100 μ g/ml) and purified E^{rn}s (1 μ g/ml). After 6 h at 37°C, the cells were washed once with PBS, and total RNA was extracted by using RNeasy kit (Qiagen) according to the manufacturer's protocol including a DNase treatment step. Reverse transcription (RT) was performed by using a cDNA synthesis kit (Promega) according to the manufacturer's instructions in a reaction volume of 30 μ l containing 1 μ g of total RNA, 0.75 μ g of oligonucleotide dT, and 30 U of avian myeloblastosis virus reverse transcriptase and incubated at 42°C for 1 h, followed by 5 min at 99°C. Then, 2 μ l of resulting cDNA was amplified by 25 cycles by using a standard PCR protocol with each cycle consisting of 45 s at 94°C, 45 s at 66°C, and 45 s at 72°C, followed by 10 min at 72°C. The primers used to amplify bovine IFN- β mRNA to produce a 571-bp product were 5'-TCACTCTGCAAACCCTTGAA-3' (sense) and 5'-GTCCAGGCACACCTGTGTA-3' (antisense); the primers used for β -actin mRNA (270-bp product) were 5'-CCAGACAGCACTGTGTTGGC-3' (sense) and 5'-GAGAAGCTGTGCTACGTCGC (antisense). The PCR for β -actin mRNA was performed as for IFN- β mRNA, but the annealing temperature was 55°C.

dsRNA-binding assays. Poly(rI)-poly(rC)-agarose beads (200 μ l) (Amersham Biotech and Sigma) were used in a 1-ml column (MoBiTec Columns; Biosciences Services, Cramlington, United Kingdom) and washed three times with binding buffer (20 mM sodium phosphate buffer [pH 7.2] containing 150 mM NaCl). A total of 2 μ g of purified wild-type or mutant E^{rns} glycoproteins diluted in 500 μ l of binding buffer was applied to the columns. The columns were washed with binding buffer and eluted in 500- μ l fractions with a stepwise gradient of increasing concentrations of NaCl (from 0.2 to 1.0 M) in 20 mM sodium phosphate buffer (pH 7.2). Dilutions of each fraction were coated onto a 96-well enzyme-linked immunosorbent assay (ELISA) plate at 4°C overnight and then blocked with 5% skimmed milk in PBS-T. Eluted E^{rns} was detected by using an anti-V5 antibody (a generous gift from R. Randall, University of St. Andrew's, St. Andrew's, Scotland).

The binding of full-length and C-terminally truncated mutants of E^{rns} to poly(rI)-poly(rC) was also analyzed by using radiolabeled recombinant proteins. [³⁵S]methionine-labeled E^{rns} was generated from pcDNA6/CD33-E^{rns}/V5-His by using the T7 TNT-quick coupled transcription-translation system (Promega). In a standard reaction, 1 μ g of template DNA and 1 μ l of [³⁵S]methionine (10 μ Ci/ μ l, >1,000 Ci/mmol [Redivue; Amersham]) was used in a final volume of 20 μ l under conditions specified by the manufacturer. Truncations were generated by restriction enzyme digestion of aliquots of pcDNA6/CD33-E^{rns}/V5-His with *Afl*III (E^{rns} [residues 1 to 66], polyprotein residues 271 to 336), *Hinc*II (E^{rns} [residues 1 to 104], polyprotein residues 271 to 374), *Apo*I (E^{rns} [residues 1 to 142], polyprotein residues 271 to 412), *Bsg*I (E^{rns} [residues 1 to 165], polyprotein residues 271 to 435), *Ava*I (E^{rns} [residues 1 to 207], polyprotein residues 271 to 477), or *Xba*I (full-length E^{rns} [residues 1 to 227], polyprotein residues 271 to 497), and these proteins were similarly synthesized. Since the E^{rns} protein synthesized from pcDNA6/CD33-E^{rns}/V5-His contains an N-terminal CD33 tag, the effect of removing this sequence on dsRNA binding was also examined. To do this, a full-length E^{rns} fragment (amino acids 1 to 227) was subcloned into pT7 β plink (25); this plasmid was cut with all of the above restriction enzymes, and the resulting linearized plasmids were also expressed in the T7 TNT quick-coupled transcription-translation system. To prepare the poly(rI)-poly(rC)-agarose for binding assays, 250 μ l of fresh beads was washed three times in 750 μ l of binding buffer (100 mM KCl, 50 mM Tris [pH 7.4], 5 mM MgCl₂, 0.1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 30 μ g of leupeptin/ml, 5 μ g of aprotinin/ml, 5 μ g of pepstatin A/ml) and resuspended as a 50% slurry in binding buffer. To determine dsRNA binding, 2 μ l of a TNT reaction mixture containing [³⁵S]methionine-labeled recombinant protein was incubated with 10 μ l of poly(rI)-poly(rC)-agarose bead slurry, followed by incubation at room temperature for 1 h in 100 μ l of binding buffer plus 1 mM dithiothreitol with gentle mixing before three washes in 750 μ l of binding buffer. Where indicated, the prewash, binding, and postbinding wash steps were performed in the presence of increased concentrations of KCl. Bound proteins were eluted from beads in 20 μ l of SDS-PAGE buffer, separated by SDS-PAGE, and visualized by autoradiography.

RNase activity assay. Purified wild-type and mutant E^{rns} glycoproteins were diluted either in RNase assay buffer (20 mM sodium acetate buffer [pH 4.5]) or cell culture medium (DMEM containing 2% FCS). The glycoproteins in cell culture medium were further incubated at 37°C for 18 h, and the RNase activity was determined as described previously (15) with slight modification. Briefly, the assay mixture (25 μ l in total) contained 0.2 μ g of wild-type or mutant E^{rns}, 12.5 μ g of yeast 16-23S RNA (Worthington), and 40 U of RNase inhibitor (RNasin; Promega) in 20 mM sodium acetate buffer (pH 4.5). The mixture was incubated at 37°C for 45 min, and the enzyme reaction was stopped by acid precipitation with 5 μ l of 25% (vol/vol) HClO₄ containing 0.75% (wt/vol) uranyl acetate. After cooling on ice for 10 min, the reaction was centrifuged for 10 min at 10,000 \times g, and the absorbance at 260 nm of the supernatant was measured.

Preparation of dsRNA. Synthetic dsRNA poly(rI)-poly(rC) (Sigma or Amersham Biosciences) was prepared at 1 mg/ml in water or PBS. An alternative dsRNA preparation was made by using the Litmus 28i plasmid (New England Biolabs) containing only its polylinker site (140 nucleotides). Litmus 28i plasmid DNA was digested in two separate reactions with either *Bgl*II or *Sna*I restriction enzymes, and transcription of equal amounts both linearized templates was carried out in a single reaction by using the RiboMAX system (Promega) according to the manufacturer's instructions. The resulting RNA was treated with RQ1 RNase-free DNase (Promega) at a concentration of 1 U per μ g of template DNA for 15 min at 37°C. After incubation, the RNA was extracted with 1 volume of water-saturated phenol-chloroform-isoamyl alcohol (25:24:1) and then precipitated with 2.5 volumes of 100% ethanol. The resulting RNA pellet was washed with 70% ethanol, dried, and suspended in water. The transcribed RNA was incubated with S1 nuclease (Promega) at a concentration of 10 U per μ g of RNA for 20 min at 25°C, and the dsRNA was recovered by ethanol precipitation.

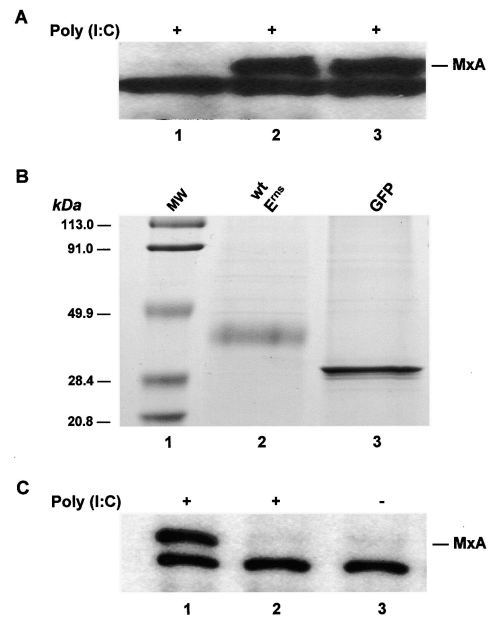


FIG. 2. Effect of E^{rns} on the induction of MxA by poly(rI)-poly(rC). (A) Pools of MDBK cells expressing E^{rns} (lane 1), E2 (lane 2), or β -galactosidase (lane 3) were treated with maintenance medium (DMEM plus 2% FCS) supplemented with 5 μ g of the synthetic dsRNA, poly(rI)-poly(rC), per ml. After 18 h the cells were harvested in sample buffer and then subjected to SDS-PAGE and immunoblot analysis with rabbit antiserum against human MxA. (B) SDS-PAGE of purified E^{rns} and GFP/V5-His. Prestained molecular mass standards (lane 1), 5 μ g of E^{rns} (lane 2), and 5 μ g of GFP/V5-His (lane 3). The gel was stained with brilliant blue R (Sigma). (C) Confluent monolayers of CTE cells were rinsed and replaced with medium either supplemented with 5 μ g of poly(rI)-poly(rC)/ml or not and then treated with 1 μ g of control protein (GFP/V5-His)/ml (lane 1), 1 μ g of E^{rns}/ml (lane 2), or mock treated (lane 3). After 18 h, the cells were harvested in sample buffer and analyzed for MxA expression. Lanes marked “+” in panels A and C indicate treatment with poly(rI)-poly(rC).

dsRNA degradation assay. Purified wild-type or mutant E^{rns} glycoproteins (1 μ g) were incubated with poly(rI)-poly(rC) (5 μ g) or in vitro-synthesized dsRNA (5 μ g) either in cell culture medium (DMEM containing 2% FCS) for 18 h at 37°C or in sodium acetate buffer (20 mM [pH 4.5] containing 150 mM NaCl) for 1 h at 37°C. The samples were analyzed on a 1.8% agarose gel, and the dsRNA was stained with ethidium bromide (0.2 μ g/ml).

RESULTS

Inhibition of dsRNA-dependent gene expression by E^{rns}. In order to study a possible role for E^{rns} in the induction of IFN and IFN-stimulated genes, stably transfected pools of MDBK cells expressing approximately similar levels of either wild-type E^{rns} or control proteins E2 and β -galactosidase (data not shown) were established (see Materials and Methods). These pools were exposed to the synthetic dsRNA, poly(rI)-poly(rC), added directly to the medium at 5 μ g/ml, and the expression levels of MxA were determined. As shown in Fig. 2A, MxA was inducible by poly(rI)-poly(rC) in cell pools expressing E2 or β -galactosidase; however, in contrast, no MxA was observed after treatment with poly(rI)-poly(rC) in cells that expressed E^{rns}.

We next examined whether the addition of exogenous recombinant E^{rns} produced in insect cells and purified to ca. 95%

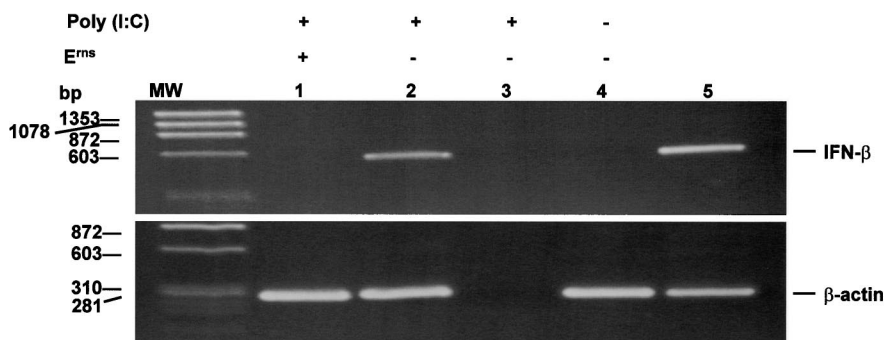


FIG. 3. Induction of IFN- β mRNA by dsRNA is inhibited by E^{rns}. CTe cells were either treated with 100 μ g of poly(rI)-poly(rC)/ml (lanes 1, 2, and 3) or mock treated (lane 4) in the presence (lane 1) or absence (lanes 2, 3, and 4) of 1 μ g of E^{rns}/ml. After 6 h RNA was extracted from the cells, reverse transcribed with oligonucleotide (dT) as a primer, and amplified by PCR with primers specific for bovine IFN- β mRNA (upper panel) or β -actin mRNA (lower panel). Lane 3 shows the absence of a PCR product from poly(rI)-poly(rC)-treated cells with no RT step. Lane 5 shows the PCR products of a bovine IFN- β cDNA clone or a bovine β -actin cDNA clone. The respective DNA mobilities of IFN- β and β -actin are indicated on the right-hand sides of each panel.

homogeneity (Fig. 2B) could inhibit the expression of MxA induced by treatment of CTe cells with poly(rI)-poly(rC). The cells were treated with a mixture of poly(rI)-poly(rC) and E^{rns}, and after overnight incubation at 37°C, cell lysates were examined for MxA expression. The results (Fig. 2C) demonstrated that the addition of E^{rns} blocks the induction of MxA by poly(rI)-poly(rC), whereas the addition of green fluorescent protein (GFP) (a control protein) had no effect on MxA induction. A minimum of 0.25 μ g of E^{rns}/ml was required to inhibit MxA induction by 5 μ g of poly(rI)-poly(rC)/ml (data not shown). In addition, MxA induction by an alternative *in vitro*-transcribed dsRNA was also inhibited by the presence of E^{rns} in the medium (see Fig. 9C). These results indicate that soluble E^{rns} prevents MxA expression in response to dsRNA and that this effect is not cell type specific.

Although MxA synthesis may be induced directly by virus infection, this activation appears to be inefficient compared to its induction by IFN- α/β (5, 34); thus, the E^{rns}-mediated inhibition of the poly(rI)-poly(rC) effect is likely to be acting indirectly through the inhibition of the intermediate production of IFN- β . To establish this, RT-PCR was used to analyze IFN- β mRNA production. As shown in Fig. 3 (upper panel), cells stimulated with poly(rI)-poly(rC) alone showed a clear IFN- β mRNA signal in contrast to cells treated with poly(rI)-poly(rC) in the presence of E^{rns} when no IFN- β signal was seen. Figure 3 (lower panel) shows that β -actin mRNA was detected in all RT reactions, indicating that all preparations contained similar amounts of RNA. These results clearly demonstrated that dsRNA induction of IFN- β mRNA synthesis was inhibited in the presence of E^{rns}. Consistent with this, E^{rns} also blocked the activation of an IFN- β promoter-luciferase reporter construct in response to the addition of poly(rI)-poly(rC) to the medium (data not shown).

Inhibition of dsRNA-dependent NF- κ B activation by E^{rns}. An early response of cells to treatment with dsRNA is to induce the nuclear translocation of the transcription factor NF- κ B and promote its DNA-binding activity in nuclear extracts; these events are important for the activation of IFN- β gene transcription. The effect of purified E^{rns} on NF- κ B activation was examined in CTe cells and human MG-63 cells. As shown in Fig. 4 cells infected with SFV or treated with TNF- α

clearly activated NF- κ B in the presence or absence of E^{rns}. In contrast, although cells treated with poly(rI)-poly(rC) activated NF- κ B in the absence of E^{rns}, the presence of E^{rns} completely blocked NF- κ B activation. These results show that the inhibition of activation of NF- κ B by E^{rns} is specific to dsRNA as the inducing signal. Furthermore, they indicate that the inhibition is not restricted to bovine cells.

RNase activity of E^{rns} is essential for the inhibition of dsRNA-dependent signaling. Histidine 297 of the CSFV polyprotein (300 in BVDV; Fig. 1) has been shown to be essential for RNase activity of CSFV E^{rns} (11, 23). In order to determine the importance of the RNase activity of BVDV glycoprotein E^{rns} to its ability to inhibit the signaling by dsRNA, we engineered a histidine-to-lysine substitution at amino acid 300 in BVDV (E^{rns}H300K). We first established whether this mutated protein has lost the RNase activity of wild-type BVDV E^{rns}. E^{rns}H300K was expressed in insect cells and purified to homogeneity as assessed by SDS-PAGE analysis (Fig. 5A). The RNase activity of the mutant glycoprotein was determined, and as shown in Fig. 5B, E^{rns}H300K is devoid of RNase activity. Next, we analyzed the stability and enzymatic activity of the wild-type and mutant proteins. Incubation of wild-type and mutant E^{rns} in tissue culture medium at 37°C for 18 h altered neither the stability of mutant or wild-type protein (both remained intact after incubation in medium) nor the enzymatic activity of wild-type E^{rns} (Fig. 5B).

We next investigated whether E^{rns}H300K could block dsRNA-dependent signaling. When this protein was added to the medium of CTe cells, it was unable to block the induction of MxA in response to poly(rI)-poly(rC), in contrast to wild-type E^{rns} (Fig. 5C). A similar failure of E^{rns}H300K to block dsRNA-dependent signaling was also seen for NF- κ B induction (data not shown). We conclude that the histidine residue at position 300 in the E^{rns} polypeptide, crucial for RNase activity, is critically required to block dsRNA-dependent signaling responses in cultured cells.

E^{rns} binds to dsRNA-agarose. Since E^{rns} efficiently blocks dsRNA signaling, we sought to establish whether BVDV E^{rns} can bind to dsRNA. Accordingly, E^{rns} expressed in S2 cells was examined for its ability to bind to poly(rI)-poly(rC)-linked agarose. E^{rns} was applied to a bed of poly(rI)-poly(rC)-agarose

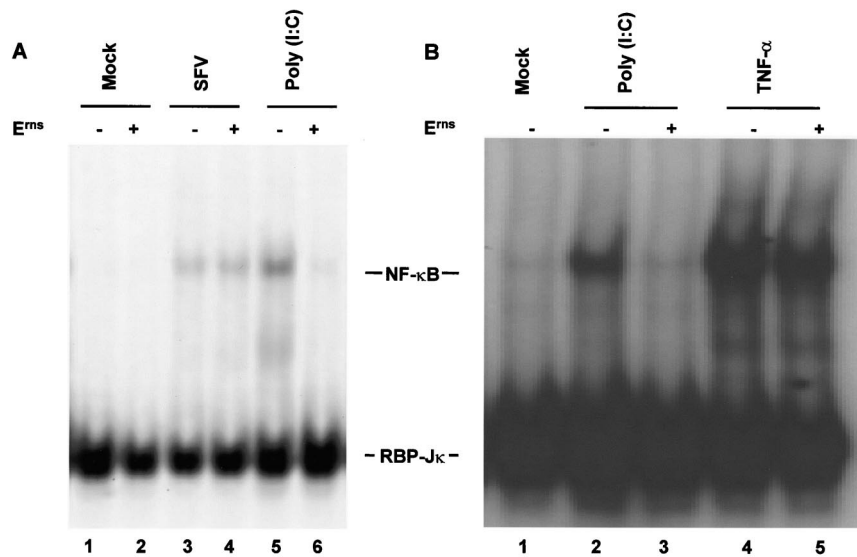


FIG. 4. E^{rns} treatment prevents NF- κ B activation by dsRNA. (A) CTe cells were either mock treated (lanes 1 and 2), infected with SFV (~ 10 PFU/cell) (lanes 3 and 4), or treated with 5 μ g/ml of poly(rI)-poly(rC) (lanes 5 and 6) for 2 h in the presence or absence of E^{rns} . (B) MG-63 cells were either mock treated (lane 1) or treated with 5 μ g of poly(rI)-poly(rC)/ml (lanes 2 and 3) or 10 ng of TNF/ml (lanes 4 and 5) for 2 h in the presence or absence of E^{rns} . Nuclear extracts were prepared from each experiment and analyzed by EMSA with the PRD II probe from the human IFN- β gene (31). The mobilities of the DNA-protein complexes are indicated on the sides of each panel. Lanes marked “+” in panels A and B indicate treatment with E^{rns} (1 μ g/ml).

and bound E^{rns} was eluted by stepwise increased concentrations of NaCl of up to 1 M. Eluted E^{rns} was detected by ELISA. The results (Fig. 6A) show that E^{rns} bound with greater affinity to poly(rI)-poly(rC)-agarose than it did to underivatized agarose, with a substantial proportion eluting at concentrations of ≥ 0.25 M NaCl.

We also examined the ability of poly(rI)-poly(rC)-agarose to bind to recombinant E^{rns} produced in reticulocyte lysates. Figure 6B shows that a significant proportion of E^{rns} remained associated with the matrix at a concentration of at least 0.3 M NaCl (Fig. 6B, upper panel) in agreement with the result obtained with E^{rns} expressed in insect cells.

Identification of amino acids involved in dsRNA binding by E^{rns} . We next took advantage of the ability of recombinant protein produced in reticulocyte lysates to bind to poly(rI)-poly(rC)-agarose to map regions of E^{rns} involved in dsRNA binding. A series of C-terminal truncations were produced (see Materials and Methods) and examined for their ability to bind to poly(rI)-poly(rC)-agarose. Truncations that retained only 143 amino acids of E^{rns} , terminating at K412 of the polyprotein, showed no loss of binding (data not shown), but only residual binding was seen with further truncation to produce a 104-amino-acid polypeptide, terminating at G375 ($E^{rns}\Delta 375$; Fig. 6B). In addition, we were able to show that the dsRNA-binding functions were not influenced by the presence of the CD33 tag at the N terminus of the E^{rns} truncations (see Materials and Methods). These results indicate that the N-terminal region of E^{rns} contains residues that influence dsRNA binding; however, when we tested the effects of KCl concentration on binding, it was clear that the $E^{rns}\Delta 375$ truncation bound less stably (Fig. 6B, lower panel) than full-length E^{rns} . Thus, there are amino acids influencing binding to poly(rI)-

poly(rC) resident in the C-terminal 123 amino acid residues of E^{rns} .

Several dsRNA-binding proteins from a wide variety of organisms interact with dsRNA through basic amino acid residues (8, 32, 33). The E^{rns} glycoprotein contains two clusters of basic amino acids: 409 KKGK 412 and 483 KKLENKSK 490 . The latter has been shown to be responsible for binding to cell surface glycosaminoglycans (15). Both of these basic clusters are present in the C-terminal 123 amino acids of E^{rns} , the region that was implicated above in modulating the affinity of binding to dsRNA. To investigate the contribution of these clusters to binding dsRNA by E^{rns} , we examined a series of single (K409A, K410T, and K412G) amino acid substitutions and a double substitution (K409A-K410T) in wild-type E^{rns} and the deletion mutant $E^{rns}\Delta 483-497$ from which the 483 KKLENKSK 490 motif had been removed (15). These proteins, expressed in *Drosophila* S2 cells and purified by chelating chromatography, were tested for dsRNA-binding activity by using poly(rI)-poly(rC)-agarose. Figure 7 shows that there are marked differences in the dsRNA-binding abilities of various mutant glycoproteins. $E^{rns}\Delta 483-497$ and the substitution mutants E^{rns} K410T, E^{rns} K412G, and E^{rns} H300K behave like wild-type E^{rns} , whereas E^{rns} K409A and the double-substitution mutant E^{rns} K409A-K410T were deficient in dsRNA binding. The reduced ability of the mutant glycoprotein E^{rns} K409A to bind to dsRNA is consistent with the lowering of affinity for dsRNA associated with the deletion of these residues from the studies by using protein produced by in vitro translation. The cluster of basic amino acid residues at the C terminus (483 KKLENKSK 490), previously implicated in the binding of E^{rns} to glycosylaminoglycans (15), appears not to contribute in dsRNA- E^{rns} binding.

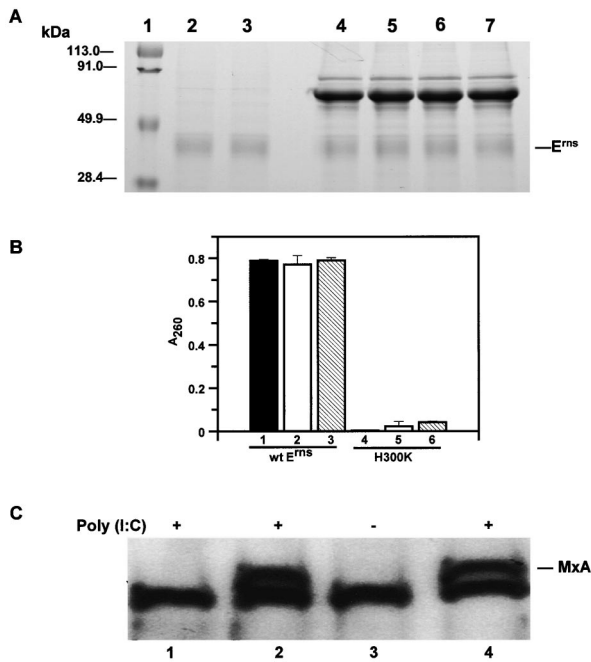


FIG. 5. Mutation of amino acid residue H300K impairs both the RNase activity of E^{rn}s and the inhibitory effect of E^{rn}s on dsRNA-induced MxA expression. (A) Stability of wild-type and mutant E^{rn}s as determined by SDS-PAGE. Prestained molecular mass standards (lane 1), 1 μ g of wild-type E^{rn}s (lane 2), 1 μ g of H300K mutant (lane 3), 1 μ g of wild-type E^{rn}s in maintenance medium (lanes 4 and 5), and 1 μ g of H300K mutant in maintenance medium (lanes 6 and 7) are shown. The samples in lanes 5 and 7 were incubated at 37°C for 18 h before electrophoresis. The gel was stained with Coomassie brilliant blue R250. (B) Stability of RNase activity of E^{rn}s. Purified wild-type and H300K mutant E^{rn}s (bars 1 and 4) and wild-type and mutant E^{rn}s incubated in cell culture medium (DMEM plus 2% FCS) for either 10 min at room temperature (bars 2 and 5) or for 18 h at 37°C (bars 3 and 6) were assayed for RNase activity at pH 4.5. The final assay mixture (25 μ l) contained wild-type E^{rn}s and mutant H300K glycoproteins (0.2 μ g), yeast 16-23S RNA (12.5 μ g), and RNase inhibitor (RNasin; 40 U) as described in Materials and Methods. The release of acid-soluble RNA was determined by measuring the absorbance at 260 nm. (C) Comparison of dsRNA-induced MxA inhibition by wild-type E^{rn}s and H300K mutant. Confluent monolayers of cells were rinsed with maintenance medium and replaced with medium containing 5 μ g of poly(rI)-poly(rC)/ml and 1 μ g of wild-type E^{rn}s/ml (lane 1), 5 μ g of poly(rI)-poly(rC)/ml and 1 μ g of H300K mutant/ml (lane 2), and 5 μ g of poly(rI)-poly(rC)/ml (lane 4). Lane 3, mock treated. Cells were incubated at 37°C for 18 h and subjected to immunoblot analysis for the MxA protein.

Although these results indicated that E^{rn}s is able to bind to a matrix of dsRNA, we also sought to determine whether E^{rn}s was also able to bind to ssRNA or dsDNA. The results of experiments set up to examine binding of E^{rn}s to poly(C)-agarose or DNA-agarose were inconclusive; E^{rn}s bound strongly and irreversibly both to poly(rC)-agarose and to dsDNA-agarose and failed to elute when it was washed with 1 M NaCl in a batch procedure. This finding contrasted with binding to poly(rI)-poly(rC)-agarose, in which bound E^{rn}s can be eluted and none remained bound to the matrix. We cannot therefore be sure that binding to these other matrices represents a specific or nonspecific binding. We have also investigated the binding of E^{rn}s to ssRNA in a nitrocellulose filter-

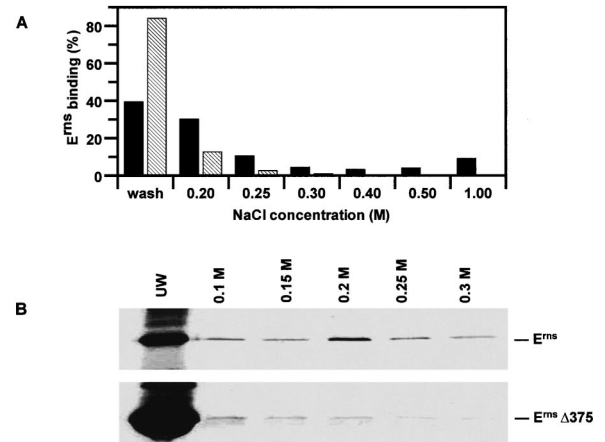


FIG. 6. Binding of E^{rn}s to poly(rI)-poly(rC)-agarose. (A) Purified H300K mutant E^{rn}s (2 μ g) was applied to a 100- μ l Minispin column containing either poly(rI)-poly(rC)-agarose (■) or agarose (▨). The columns were equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl, and the E^{rn}s was eluted with steps of increasing concentrations of NaCl (0.2 to 1 M) in 20 mM sodium phosphate buffer (pH 7.2). The E^{rn}s concentration in each fraction was determined by ELISA as described in Materials and Methods. (B) [³⁵S]methionine-labeled full-length E^{rn}s (upper panel) or E^{rn}s Δ 375 (lower panel) was incubated with poly(rI)-poly(rC)-agarose beads and washed at increasing ionic strengths as described in Materials and Methods and as indicated on the top of each lane. The beads were then treated with polyacrylamide gel sample buffer, and released protein was separated by SDS-PAGE and visualized by autoradiography.

binding assay. Evidence for E^{rn}s binding to ssRNA was seen only for mutants that lacked RNase activity (H300K and K412G [see below]; also data not shown). We presume that the RNase activity of the E^{rn}s destroys the ssRNA. Our results on binding of E^{rn}s to poly(rI)-poly(rC)-agarose, however, show differences between mutants, and these mutants were investigated further.

E^{rn}s mutants lack the ability to block dsRNA-dependent signaling. To determine the effects on dsRNA signaling of the substitutions in the two basic amino acid motifs, cells were exposed to dsRNA in the presence of wild-type or mutant forms of E^{rn}s. Figure 8A shows that E^{rn}s Δ 483-497 and E^{rn}sK410T behaved like wild-type E^{rn}s in inhibiting the induction of MxA by dsRNA. In contrast, the double substitution E^{rn}sK409A-K410T, or the single substitutions E^{rn}sK409A or E^{rn}sK412G, were unable to block dsRNA-dependent signaling.

The results presented above correlate E^{rn}s binding to poly(rI)-poly(rC)-agarose with its ability to block dsRNA-dependent signaling. However, they do not account for the properties of the E^{rn}sK412G protein, since it is able to bind to poly(rI)-poly(rC)-agarose and yet has lost the ability to inhibit dsRNA-dependent signaling. Since we also saw a loss of dsRNA signaling inhibition associated with the RNase-deficient E^{rn}s H300K protein (Fig. 5B), we reasoned that the loss of signaling inhibition by the E^{rn}sK412G protein could be due to a similar loss of RNase activity or, alternatively, the mutant E^{rn}s glycoproteins may not be stable. To test these possibilities, two series of experiments were carried out.

First, as described above for wild-type E^{rn}s, wild-type and mutant E^{rn}s was mixed with tissue culture maintenance me-

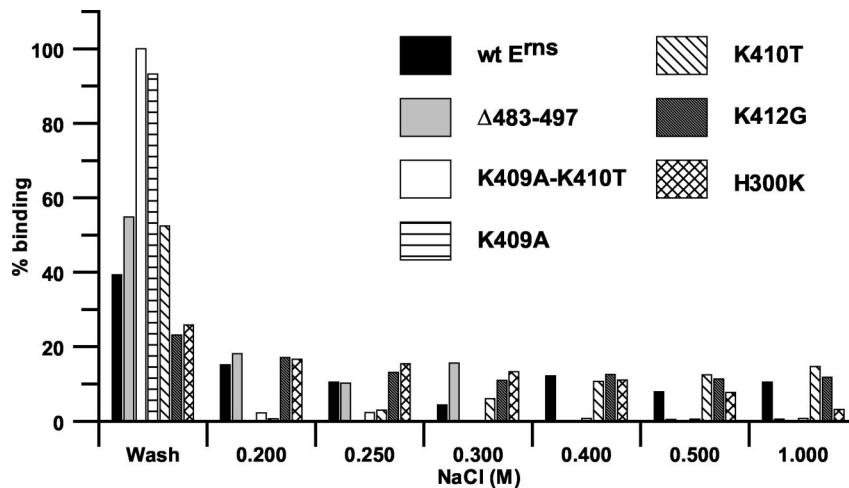


FIG. 7. Binding of mutant E^{rns} glycoprotein to poly(rI)-poly(rC)-agarose. E^{rns} mutant glycoproteins expressed in *Drosophila* S2 cells were purified by chelating chromatography (see Materials and Methods) and applied to poly(rI)-poly(rC)-agarose columns. The binding of mutant glycoproteins to poly(rI)-poly(rC)-agarose was analyzed as described in Fig. 6A legend.

dium and incubated for 18 h at 37°C. The results showed no degradation of E^{rns} after incubation (Fig. 8B). Second, RNase assays were performed on each of the purified recombinant E^{rns} proteins, either as purified E^{rns} or as purified E^{rns} added to tissue culture maintenance medium and incubated for 18 h at 37°C. The results showed that the mutant glycoproteins E^{rns}K409A-K410T and E^{rns}K409A and the deletion mutant E^{rns}Δ483-497 showed RNase activity similar to the wild-type E^{rns}, which was not lost upon incubation at 37°C (Fig. 8C). In contrast, E^{rns}K412G was completely devoid of RNase activity (Fig. 8C), a finding comparable to the activity seen with the RNase-negative mutant glycoprotein E^{rns}H300K (Fig. 5B).

E^{rns} degrades dsRNA at low pH. The results presented above demonstrate that both the binding activity of E^{rns} to dsRNA-agarose and the RNase activity of E^{rns} correlate with the inhibition of dsRNA-dependent signaling. One explanation for the ability of E^{rns} to inhibit dsRNA-dependent signaling is that the dsRNA in the cell culture medium is targeted for degradation by the RNase activity, although it should be stressed that previous reports indicate that ssRNA is the preferred substrate for the RNase activity of E^{rns}, with dsRNA being degraded inefficiently (37). To test whether the dsRNA is degraded by E^{rns}, poly(rI)-poly(rC) was incubated in the cell culture medium at 37°C for 1 h and analyzed by agarose gel electrophoresis. As shown in Fig. 9A, poly(rI)-poly(rC) in tissue culture medium was degraded by the addition of E^{rns}. However, we considered that the poly(rI)-poly(rC) preparations may contain regions of ssRNA, which could be digested by E^{rns}.

To investigate more carefully the degradation of dsRNA by E^{rns}, we synthesized a 140-bp dsRNA by in vitro transcription and treated this product so as to remove all ssRNA structures (see Materials and Methods). The synthetic dsRNA retained the ability to induce dsRNA-dependent signaling events and is inactivated by the coaddition of E^{rns} to the cell culture medium (Fig. 9C). When this dsRNA product was incubated with wild-type E^{rns} in the cell culture medium for 18 h at 37°C, it remained intact (Fig. 9B, upper panel), unlike the result seen

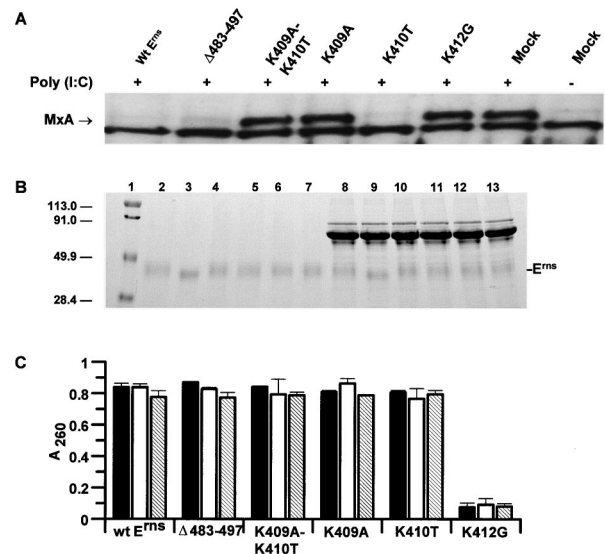


FIG. 8. Functional analysis of E^{rns} mutants. (A) Inhibition of MxA induction by dsRNA. CTe cells were either mock treated or treated with 5 μg of poly(rI)-poly(rC)/ml in maintenance medium in the presence or absence of wild-type or mutant E^{rns} glycoproteins (1 μg/ml) as indicated. After incubation at 37°C for 18 h, MxA induction was analyzed by immunoblotting. Lanes marked “+” indicate that the cells were treated with poly(rI)-poly(rC). (B) Comparison of the protein stability of wild-type and mutant E^{rns}. Wild-type and mutant E^{rns} was purified and added to maintenance medium (DMEM plus 2% FCS) and incubated for 18 h at 37°C, and samples containing 1 μg of E^{rns} were taken for electrophoresis. Lanes 2 to 7, purified E^{rns}; lanes 8 to 13, E^{rns} in maintenance medium following incubation. Lane 1, prestained markers; lanes 2 to 8, wild-type E^{rns}; lanes 3 and 9, deletion mutant Δ483-497; lanes 4 and 10, double mutant K409A-K410T; lanes 5 and 11, mutant K409A; lanes 6 and 12, mutant K410T; and lanes 7 and 13, mutant K412G. Following electrophoresis the gel was stained with Coomassie brilliant blue R250. (C) RNase activity assays of purified wild-type (wt) E^{rns} and E^{rns} mutants with the indicated substitutions in the E^{rns} sequence. The glycoproteins were either incubated in 20 mM sodium acetate buffer (pH 4.5) (■), in cell culture medium (DMEM plus 2% FCS) for 10 min at room temperature (□), or in cell culture medium for 18 h at 37°C (▨). RNase assays were performed at pH 4.5 as described in Materials and Methods. Bars show the standard deviations of triplicate datum points.

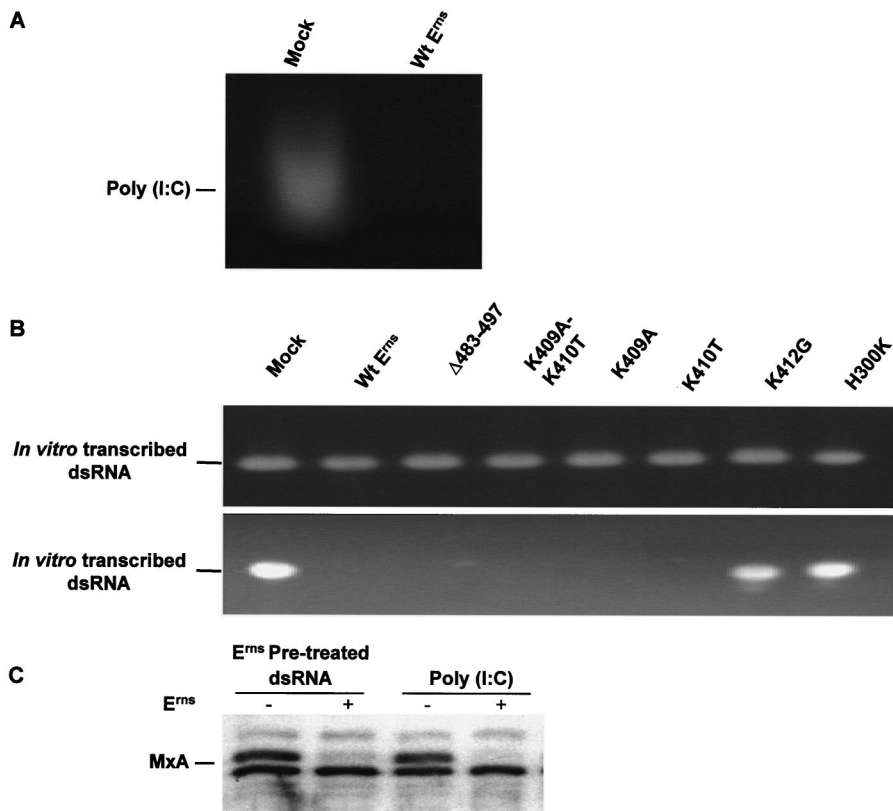


FIG. 9. Effect of E^{rns} on dsRNA stability at neutral and acidic pH conditions. (A) Synthetic dsRNA poly(rI)-poly(rC) (5 μ g) was either mock treated or treated with wild-type E^{rns} (0.2 μ g) in 50 μ l of cell culture medium (1 \times DMEM plus 2% FCS) at 37°C for 18 h. (B) In vitro-transcribed dsRNA (5 μ g) was either mock treated or treated with wild-type E^{rns} or the indicated E^{rns} mutants (0.2 μ g). The digestion was carried out either in 50 μ l of cell culture medium (1 \times DMEM plus 2% FCS) at 37°C for 18 h (upper panel) or 50 μ l of sodium acetate buffer (20 mM [pH 4.5], containing 150 mM NaCl) for 1 h at 37°C (lower panel). The samples were analyzed on a 1.8% agarose gel, and the RNA was stained with ethidium bromide. (C) The induction of MxA by dsRNA previously incubated with E^{rns}. RNA was produced by in vitro transcription and incubated with E^{rns} at 37°C for 18 h, followed by phenol-chloroform extraction and ethanol precipitation. CTe cells were rinsed, and the medium was replaced with maintenance medium (DMEM plus 2% FCS) with or without wild-type E^{rns} (1 μ g/ml) and equal amounts (5 μ g/ml) of either E^{rns}-pretreated in vitro-transcribed dsRNA or poly(rI)-poly(rC) not treated with E^{rns}. The cells were incubated at 37°C for a further 18 h, and MxA levels were analyzed by immunoblotting. Lanes marked “+” indicate the samples incubated with both RNA and E^{rns}.

above with poly(rI)-poly(rC). From these results, we deduced that the integrity of dsRNA was not affected by E^{rns} in the tissue culture medium. Furthermore, when this dsRNA was recovered from the medium after incubation with E^{rns} and any associated proteins were removed by phenol extraction, this dsRNA retained its ability to stimulate dsRNA-dependent signaling in cells, and yet this activation was inhibited by the addition of E^{rns} to the medium (Fig. 9C).

Although these results indicate that, under the conditions extant, E^{rns} does not degrade dsRNA in the cell culture medium, we note that the conditions of the cell culture medium (pH 7.2 to 7.4) are not optimal for the RNase activity of E^{rns}, pH 4.5 to 5.5 (8; unpublished results)—the pH range of several compartments of the cell. Experiments were carried out to examine whether E^{rns} can degrade dsRNA at pH 4.5. The 140-bp synthetic dsRNA produced in vitro was mixed with wild-type E^{rns} in either acidic conditions (pH 4.5) or in cell culture medium. Figure 9B (lower panel) shows that the synthetic dsRNA was completely degraded in acidic conditions (pH 4.5) by E^{rns} in contrast to its stability in cell culture medium. We also investigated the dsRNase activities of mutant

forms of E^{rns}. In addition to the wild-type E^{rns}, E^{rns}K409A-K410T, E^{rns}K409A, and E^{rns}K410T and the deletion mutant E^{rns} Δ 483-497 retained dsRNase activity, whereas E^{rns}K412G and E^{rns}H300K were unable to degrade dsRNA under the conditions tested (Fig. 9B, lower panel). These results show that E^{rns} can degrade dsRNA in acidic conditions and suggest that this activity may manifest in a low-pH compartment of the cell.

DISCUSSION

The innate immune response generated against viral infections is predominantly mediated by IFNs whose induction is triggered by dsRNA produced as a consequence of viral replication (16). Although most of this dsRNA is intracellular, it has been hypothesized that virally induced cell death can result in the release of dsRNA, and it is this material that is thought to be the ligand for Toll-like receptor 3 (2). However, direct evidence for the release of dsRNA from virus-infected cells is very sparse, influenza virus-infected cells being the only example known to the authors (21). Nevertheless, the peculiar prop-

erty of pestiviruses to encode a secreted glycoprotein with RNase activity (the E^{rns} product) stimulated us to examine whether it was involved in modulating cellular responses to dsRNA added directly to the cell culture medium. Our results show that bovine cells transfected with a plasmid encoding a secreted form of E^{rns} no longer responded to the addition of dsRNA to the culture medium; similarly, dsRNA responses were blocked by the addition of recombinant E^{rns} protein to the medium. The effect of E^{rns} on the IFN system operates at the level of blocking the IFN-inducing signal since when E^{rns} was present in the medium with the dsRNA, we saw no activation of NF- κ B DNA-binding activity, which controls the expression of the IFN- β gene (19, 31), we saw no accumulation of IFN- β mRNA, and E^{rns} blocked the activation of an IFN- β promoter-luciferase reporter construct in response to the addition of poly(rI)-poly(rC) to the medium.

The RNase activity is essential for the ability of E^{rns} to block the dsRNA-mediated signaling as evinced by the effects of mutations at amino acids 300 and 412. Since other mutations blocked dsRNA-dependent signaling without affecting RNase activity there must be additional functions of E^{rns}. We have shown that E^{rns} also binds to dsRNA-agarose, and this property correlates with the inhibition of dsRNA-dependent signaling. In contrast, GAG-binding activity was not needed for inhibition of dsRNA-induced responses. It is probable that E^{rns} degrades the dsRNA added to the cell culture medium. Although we saw no degradation of dsRNA at neutral pH, dsRNA was degraded by E^{rns} at a lower pH. From these results we suggest that dsRNA is bound by E^{rns}, but it is not degraded by E^{rns} until the complex is present at a lower pH, such as a low-pH compartment of the cell. We have sought to test this hypothesis by altering the pH of cellular compartments by the addition of 10 μ M chloroquine to the cells. The results were not conclusive since the addition of chloroquine inhibited the induction of MxA by dsRNA directly (data not shown). If E^{rns} acts in, for example, an endosomal compartment of cells, then whether E^{rns} can access this compartment becomes an issue. Recently, this issue has been addressed by Langedijk (18), who adduced that peptides corresponding to the C-terminal domain of E^{rns} were able to translocate across cell membranes and the full-length glycosylated E^{rns} was detected inside the cell, as well as on the cell surface. Thus, it is likely that E^{rns} can access low-pH compartments inside the cell.

Pestiviruses that have been engineered to lose RNase activity are viable. One study with a CSFV virus which lacked RNase activity showed that, intriguingly, it was cytopathogenic (11). The reason for this was not clear. In light of our results, we can suggest that, because treatment of cells with dsRNA can lead to apoptosis (4, 17), it may be that the cells (SK6 cells) used in the study by Hulst et al. (11) were particularly sensitive to dsRNA from cellular debris, and thus the apoptosis may have been due to dsRNA released from cells into the medium. If so, supplementing this mutant with exogenous soluble E^{rns} might have reduced cytopathogenicity.

Further studies carried out with a CSFV virus and a BVDV genotype 2 genetically modified to lack RNase activity (neither of which were reported to be cytopathogenic) showed that they were attenuated in animals (22, 23). In acute infections with these modified viruses either no viremia (in pigs infected with mutant CSFV) or markedly reduced viremia (in calves infected

with a BVDV-2 mutant) was observed after infection, and infected animals showed reduced clinical signs. To our knowledge, however, there have been no reports on whether these mutants can initiate persistent infections. Taken as a whole, it seems likely that, during either an acute or a persistent infection, these mutants will be more susceptible to the IFN response or induce an altered innate immune response *in vivo*. The results of experiments to measure IFN responses after infection of animals with these mutants would be of considerable interest.

Pestiviruses may be unique in their ability to inhibit dsRNA signaling added to the exterior of the cell, but viruses generally have a wide range of ways to control the antiviral action of IFNs. For example, viruses encode proteins that inhibit the IFN signaling pathways; examples include the V protein of paramyxoviruses that target STATs for degradation (38) and poxviruses that encode secreted proteins to act as decoys for IFN and block its action (1, 7). It seems unlikely that pestiviruses take active steps to inhibit IFN signaling in cells: cells infected with BVDV produce MxA on incubation with IFN with the same kinetics as uninfected cells (3), and the antiviral activity of IFN is not inhibited in BVDV-infected cells (30). It is becoming clear that viruses also have mechanisms to limit the yield of IFN produced by an infected cell. A common way to do this is by producing dsRNA-binding proteins (for example, influenza virus NS1 protein [33] and reovirus sigma 3 protein [32; reviewed in reference 7]), although more specific pathways may also exist (for example, the E6 protein of human papillomavirus type 16 binds to and inhibits IRF-3 function [26]).

It is not likely that E^{rns} is sufficient for the inhibition of all IFN-inducing signals produced by virus, since no effect was observed on the induction of IRF-3 DNA-binding activity in CTE cells infected with SFV in the presence of E^{rns} (data not shown). There are additional signals, and there must be additional blocks to the induction of IFN by BVDV (3). Recently, the role of N^{pro} has been associated with the ability of classical swine fever virus to inhibit IFN induction (27); whether N^{pro} acts directly to inhibit IFN- β transcription or whether it acts through another virus polypeptide is not known.

It is probable that the ability of E^{rns} to block signaling induced by the presence of extracellular dsRNA plays an important role in the ability of BVDV to sustain a persistent infection of the fetus where the innate immune response is likely to be the predominant antiviral defense of the host. During infection of the early fetus, secreted E^{rns} may act to counteract any dsRNA released by dying infected cells and thus the host fetus would fail to mount an innate antiviral response by this route. In addition, the existence of a protein with the properties of E^{rns} is strong evidence of the importance of the need for cells to respond to extracellular dsRNA in an antiviral response.

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