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Proteolytic Cleavage of the Reovirus Sigma 3 Protein Results in Enhanced Double-Stranded RNA-Binding Activity: Identification of a Repeated Basic Amino Acid Motif within the C-Terminal Binding Region

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The reovirus capsid protein σ3 was examined for double-stranded RNA (dsRNA)-binding activity by Northwestern (RNA-protein) blot analysis. Treatment of virion-derived σ3 protein with *Staphylococcus aureus* V8 protease led to an increase in the dsRNA-binding activity associated with the C-terminal fragment of the protein. Recombinant C-terminal fragments of the σ3 protein were expressed in *Escherichia coli* from the S4 cDNA of reovirus serotype 1. These truncated σ3 proteins displayed proteolytic processing and dsRNA-binding activity similar to those observed for native, virion-derived σ3 protein as measured by Northwestern blot analysis. Construction of a modified pET3c vector, pET3Exo, allowed the production of 3′-terminal deletions of the S4 cDNA by using exonuclease III and rapid screening of the induced truncated σ3 proteins. An 85-amino-acid domain within the C-terminal portion of the σ3 protein which was responsible for dsRNA-binding activity was identified. The 85-amino-acid domain possessed a repeated basic amino acid motif which was conserved in all three serotypes of reovirus. Deletion of one of the basic motifs, predicted to be an amphipathic α-helix, destroyed dsRNA-binding activity.

Sigma 3 protein is a major outer capsid protein of reovirus (50), a segmented, double-stranded RNA (dsRNA) virus (25). This 41-kDa protein, encoded by the S4 genome segment (31, 33) is removed from the virion early in the course of infection (53). Sigma 3 (σ3) protein binds to the naturally occurring A form of dsRNA, and poly(rI)-poly(rC) columns are routinely used for its purification (23, 24, 46). σ3 does not bind to single-stranded RNA or double-stranded DNA (23). There is no evidence that the dsRNA-binding activity associated with σ3 is selective for either sequence or structure-specific dsRNA targets, nor does σ3 appear to play a major role in the packaging of the progeny dsRNA genome (18). Packaging of the progeny genome dsRNA segments is concomitant with replication of the second (minus) strand of genome RNA (1, 62), and the genome dsRNA segments are at all times sequestered within a protein inner core which does not contain σ3 (20, 23). However, the dsRNA-binding activity of the σ3 protein does appear to have a role in antagonizing the host antiviral mechanisms mediated by interferon (44).

Viral infection or treatment of cells with interferon induces the expression of an RNA-dependent P1 protein kinase (39, 55). The kinase requires low concentrations of dsRNA and ATP for activation (30). Localized activation of the kinase by P1 autophosphorylation leads to subsequent phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) (30, 39). Phosphorylation of eIF-2α interferes with GDP-GTP exchange and subsequent cycling of the factor between each round of translation initiation (41). The P1/eIF-2α kinase is an important regulator of translational control (22) and a major component of the interferon-mediated antiviral state (44).

Viruses have evolved a number of different mechanisms to interfere with activation of the P1/eIF-2α kinase (44). For example, the reovirus σ3 protein is thought to block activation of the P1/eIF2α kinase by sequestering activator dsRNA molecules (24). Genetic studies have revealed that the reovirus S4 gene is also involved in both the inhibition of cellular protein synthesis (49) and the establishment of persistent infections (2). The possible roles that σ3 and the interferon-induced, RNA-dependent kinase play in the regulation of cellular protein synthesis and viral persistence in reovirus-infected cells are not yet clear.

The peptide motif(s) within σ3 responsible for the dsRNA-binding activity has not been identified. Northwestern (RNA-protein) blot analysis of proteolytic fragments of virion-derived σ3 localized the site of dsRNA-binding activity to the C-terminal 16-kDa fragment of the σ3 protein, distinct from a zinc finger motif which is present in the N-terminal portion of the protein (46). The 16-kDa (148-amino-acid) C-terminal proteolytic fragment of σ3, which binds dsRNA, does not possess any of the previously described RNA-binding motifs or display appreciable homology to other RNA-binding proteins.

As an extension of our effort to elucidate the role of dsRNA-binding proteins in the action of interferon, we have attempted to define the region(s) of the reovirus σ3 protein responsible for the dsRNA-binding activity. In the present study we show that cleavage of virion-derived σ3 protein with *Staphylococcus aureus* V8 protease led to an increase in dsRNA-binding activity of the C-terminal fragment. Truncation of the S4 cDNA and subsequent expression with an efficient, inducible bacterial system allowed the production of mutant σ3 proteins which were analyzed for dsRNA-binding activity. Recombinant σ3 displayed proteolytic processing and dsRNA-binding activity comparable with that of the virion-derived parent protein. Deletion analysis identified an 85-amino-acid region in σ3 which is sufficient for

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dsRNA binding and contained two copies of a conserved basic amino acid motif.

MATERIALS AND METHODS

Abbreviations. The abbreviations used are as follows: PCR, polymerase chain reaction; PP2A, protein phosphatase 2A; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; ExoIII, exonuclease III; SDS, sodium dodecyl sulfate; and IPTG, isopropyl-β-D-thiogalactopyranoside.

Materials. Restriction endonucleases, S1 nuclease, Klenow fragment, and T7 RNA polymerase were obtained from New England Biolabs; T4 DNA ligase and RNA ligase were from Bethesda Research Laboratories. Bluescript SK$^+$ vector DNA and T3 RNA polymerase were purchased from Stratagene. Sequenase 2.0 was from U.S. Biochemicals, and *Thermus aquaticus* DNA polymerase I was obtained from Perkin-Elmer Cetus. Oligonucleotide primers were obtained either from Stratagene (T3 and T7 primers) or synthesized by using an Applied Biosystems DNA synthesizer model 380A. [α-^{32}P]UTP (650 Ci/mmol) and ^{125}I-labeled protein A were obtained from ICN, [5′-^{32}P]pCp (3,000 Ci/mmol) was from NEN, and RQ1 DNase and RNasin were from Promega. Poly(rI)-poly(rC) was a generous gift from the Antiviral Substances Program, National Institutes of Health. Nitrocellulose filter membranes (0.45-μm pore size) were purchased from Schleicher & Schuell.

Cells and viruses. Human U cells were grown in monolayer culture in Eagle's minimal essential medium (GIBCO) supplemented with 5% fetal bovine serum (HyClone), 100 U of penicillin (GIBCO) per ml, 100 μg of streptomycin (GIBCO) per ml, and 2 mM glutamine (GIBCO). Working stocks of reovirus serotype 1 (Lang strain) were produced from mouse L-cell monolayers infected at a low multiplicity of infection with plaque-purified virus (36). Purified reovirions were prepared from suspension cultures essentially as described previously (50).

pET3-ΔS4 construction. The pET3-ΔS4 construction contains a truncated S4 insert corresponding to amino acids 184 to 365. pET3-ΔS4 was generated by PCR (42) with the SP6 primer as the 3′ primer and pGEM4-S4 (3) as the template DNA. The 5′ primer for the pET3-ΔS4 construct, ACGAATTC**ATATG**ATGAGAGACCCATCACA, included an *Eco*RI site (underlined) and an *Nde*I site (boldface type). The 5′ pET3-ΔS4 primer is homologous with the S4 gene from nt 582 to 601 (3). The PCR product was digested with *Nde*I and *Bam*HI and then ligated into the corresponding sites of the pET3c bacterial expression vector (52). The pET3-ΔS4 construction expresses a nonfusion σ3 protein, σ3(184-365), which begins at methionine residue 184 of σ3 and includes the C-terminal 182 amino acids.

pET3-V8S4 construction. The 5′ primer for the pET3-V8S4 construct, ACGAATTC**ATATG**GTTTAC/TGATTACTCT GAG, likewise included an *Eco*RI site (underlined) and an NdeI site (boldface type). The 5′ pET3-V8S4 primer is homologous with the S4 gene from nt 693 to 713. The V8S4 insert was prepared and inserted into the pET3c vector as described above for the construction of pET3-ΔS4.

pET3Exo construction. To generate a pET vector suitable for deletion analysis strategies utilizing ExoIII, the pET3c vector of Studier et al. (52) was digested at the unique *Eco*RI and *Hin*dIII sites (nt 4638 and 29, respectively) and the ends were blunted with the Klenow fragment. The linearized vector was then ligated, and pET-Δ3c colonies which lacked the *Eco*RI and *Hin*dIII sites were selected. The pET-Δ3c vector was opened at the unique *Bam*HI site (nt 510), and the ends were blunted with the Klenow fragment. A polylinker region obtained by PCR amplification of M13mp19 DNA using reverse and −20 primers was blunted with the Klenow fragment and then inserted into the blunted, phosphatase-treated pET-Δ3c vector. The desired vector, designated pET3Exo, contains the polylinker with the *Hin*dIII site adjacent to the *Nde*I site.

pET3Exo-V8S4 deletions. The V8S4 insert was directionally cloned into the *Nde*I and *Bam*HI sites of the pET3Exo vector to yield pET3Exo-V8S4. To generate 3′ deletions, pET3Exo-V8S4 plasmid DNA was digested with both *Bam*HI and *Kpn*I (or *Sac*I) and then treated for various periods of time with ExoIII. The ExoIII digestions were terminated by the addition of EDTA, treated briefly with S1 nuclease and then with the Klenow fragment, and then ligated with T4 DNA ligase. DNA derived from each of the ExoIII time points was separately transformed into BL21(DE3) pLysS bacteria. Overnight cultures representing the various time points were induced with 0.5 mM IPTG for 4 h at 37°C; whole bacterial cell extracts were prepared and analyzed on SDS–20% acrylamide gels (27). The DNA from those time points which yielded induced protein products of the desired size were transformed into the BL21(DE3) pLysS bacteria, and individual colonies were screened following induction by Western blot (immunoblot) analysis to obtain a nested set of σ3 deletion products. The pETExo-V8S4 constructs were sequenced (45) to determine the exact position of the deletion within the S4 gene.

Recombinant σ3 protein expression. *Escherichia coli* cells containing the T7 RNA polymerase as a lysogen [BL21 (DE3)] were transformed with the pET3 constructs. Selection was with ampicillin (200 μg/ml) and growth was at 37°C with shaking (275 rpm). Three milliliters of superbroth (35 g of tryptone, 20 g of yeast extract, and 5 g of NaCl per liter) containing ampicillin (500 μg/ml) was inoculated with 10 μl of overnight culture and incubated until growth was just visibly detectable. An additional 500 μg of ampicillin per ml was then added, and incubation was continued until the optical density at 600 nm reached 0.1. The cultures were transferred to Eppendorf tubes, and the bacteria were pelleted by microcentrifugation for 2 min. The cell pellets were resuspended in 4 ml of superbroth containing ampicillin (500 μg/ml) and incubated until the optical density at 600 nm was 0.2. The cultures were divided, and IPTG (1 mM final concentration) was added to one of the 2-ml cultures; both were incubated at 37°C for an additional 4 h before harvest. The bacteria were pelleted with a 2-min spin in a microcentrifuge. The copious use of ampicillin and the pelleting and resuspension of the transformed bacteria in fresh selective media were done to minimize the amount of β-lactamase present in the cultures. This increased the efficiency of ampicillin selection and led to significantly higher levels of induced σ3-derived protein.

Purification of induced σ3 protein. For whole-cell analysis, pellets from induced BL21(DE3) cultures were resuspended with sonication in 300 μl of 1× Laemmli loading buffer and boiled for 5 min prior to PAGE analysis. To obtain insoluble material, whole cell pellets were disrupted by sonication at 4°C in extraction buffer (20 mM Tris-chloride, pH 7.4 at 25°C, 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 0.5% Nonidet P-40), and homogenates were microcentrifuged for 15 min at 4°C. The pelleted insoluble material was suspended by sonication in extraction buffer, and the extraction procedure was repeated three times. The final pellet was suspended by sonication in 300 μl of 1× Laemmli loading buffer and boiled for 5 min prior to gel analysis.

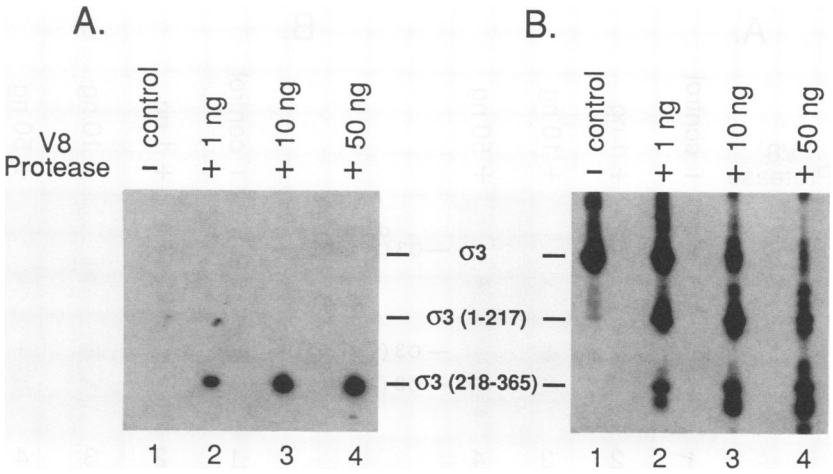

FIG. 1. Limited proteolysis of reovirus sigma 3 protein with *S. aureus* V8 protease leads to activation of dsRNA-binding activity. (A) Northwestern blot analysis of virion-derived σ3 protein fractionated on an SDS-polyacrylamide gel in the presence (+) or absence (−) of V8 protease treatment. Proteolysis was carried out in the stacking layer of the acrylamide gel. (B) Western blot of the same nitrocellulose filter used in the Northwestern blot analysis, described above, using polyclonal σ3 antisera followed by ^{125}I-labeled protein A. Conditions were as described in Materials and Methods.

Antibody production. Polyclonal antibody to the σ3 protein was raised in New Zealand White rabbits by using a TrpE-fusion protein product. The *Bam*HI-*Bam*HI insert from the pGEM4-S4 construct which contains the reovirus serotype 1 (Lang) S4 cDNA (6) was cloned into the pATH bacterial expression vector (15). The induced protein was analyzed by SDS-PAGE (27); detection was by staining with Coomassie brilliant blue R250 and independently by Western analysis with antireovirion antisera (4). The fusion protein was extracted from acrylamide gel slices and used for immunization with RIBI adjuvant (RIBI ImmunoChem Research, Inc.) as previously described (4). No detectable Western signal to reovirion proteins was obtained with the rabbit preimmune serum.

Western blots. Western immunoblotting was performed as previously described (56). Filter membranes were routinely probed with a 1:1,000 dilution of σ3 antiserum. Alternatively, σ3 antiserum immunoselected for antibodies directed at epitopes present in the σ3(221-273) recombinant protein was used at a 1:500 dilution. Selection was performed by passing σ3 antiserum sequentially over two immunoselective columns. The first column consisted of a mixture of Affi-Gel 10 and Affi-Gel 15 matrices (Bio-Rad) to which insoluble protein from induced *E. coli* BL21(DE3) containing the pET3c construct was coupled. The flowthrough fraction was loaded onto an Affi-Gel 10 matrix to which insoluble protein, σ3(221-273), from induced BL21(DE3) containing the pET3-V8S4 12-4 construct was coupled (see figure legends). The bound antibody was eluted at low pH, and the antibody fractions were neutralized. Detection of antigen-antibody complexes was with ^{125}I-labeled protein A (0.05 μCi/ml) and by autoradiography.

Northwestern blots. Proteins were separated by SDS-PAGE and electroblotted at 4°C to nitrocellulose filter membranes. The filters were then blocked for 4 to 6 h at room temperature in standard binding buffer (7) (10 mM Tris-chloride, pH 7.0, 50 mM NaCl, 1 mM EDTA) containing 0.04% (wt/vol) Ficoll 400, 0.04% (wt/vol) polyvinylpyrrolidone, and 0.04% (wt/vol) bovine serum albumin as blocking agents. Following blocking, the filters were probed for 2 h at room temperature with 5×10^4 cpm of ^{32}P-labeled dsRNA per ml (approximately 1×10^6 cpm/μg) and then rinsed for 30 min with standard binding buffer (minus blocking agents) prior to autoradiography. The probe, either poly(rI)-poly(rC) or reovirus genome dsRNA, was chromatographed on Sephadex G-50 to remove small RNA species prior to labeling with [5′-^{32}P]pCp (3,000 Ci/mmol) and RNA ligase (17). After the pCp radiolabeling reaction, the labeled dsRNA probe was chromatographed on another Sephadex G-50 column to remove unincorporated [5′-^{32}P]pCp.

Peptide mapping with V8 protease. Peptide analysis of the *S. aureus* V8 protease-generated digestion products of σ3-derived proteins was performed by following the gel digestion procedure of Cleveland et al. (12).

RESULTS

Reovirus proteins σ3, λ1, and σ2 have been shown to possess dsRNA-binding activity detectable by Northwestern blot analysis following denaturing gel electrophoresis (14, 46). However, the reovirus σ3 protein is the only viral protein present in reovirus-infected cell extracts with affinity for dsRNA under nondenaturing conditions (23, 46). Indeed, dsRNA-binding activity attributed to σ3 could also be detected in extracts prepared from reovirus-infected cells by means of a gel mobility shift assay (34). Because the σ3 protein, like the retroviral Rev protein (61), was prone to multimerization and aggregation, a gel mobility shift assay was not optimal for monitoring the dsRNA binding of mutant σ3 proteins. Therefore, the Northwestern blot assay which had been used successfully to characterize many RNA-binding proteins (7, 9, 40, 51), including σ3 (14, 46), was utilized to screen σ3 mutants for dsRNA-binding activity.

The carboxy-terminal V8 protease fragment derived from both virion and recombinant σ3 proteins retains dsRNA-binding activity. Cleavage of virion-derived σ3 from either serotype 1 (Lang) or serotype 3 (Dearing) reovirus with *S. aureus* V8 protease resulted in a substantial increase in dsRNA-binding activity which localized to the C-terminal fragment, designated σ3(218-365), as measured by Northwestern blot assay (Fig. 1A). The N-terminal portion of the protein σ3(1-217) failed to display any detectable binding

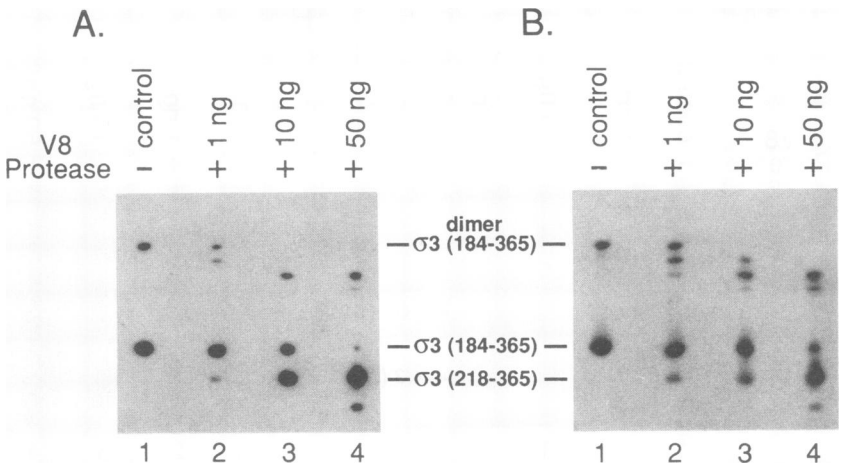

FIG. 2. Limited proteolysis of σ3(184-365) protein produced in *E. coli* from the pET3-ΔS4 construct leads to an increase in dsRNA-binding activity. (A) Northwestern blot analysis of σ3(184-365) protein fractionated on an SDS-polyacrylamide gel in the presence (+) or absence (−) of V8 protease. (B) Western blot of the same nitrocellulose filter used in the Northwestern blot analysis, described above, using polyclonal σ3 antisera followed by ^{125}I-labeled protein A. Conditions were otherwise as described for Fig. 1.

activity, as previously reported (46). To take advantage of both the V8-mediated increase in dsRNA-binding activity of σ3 and an expression system readily amenable to mutagenesis procedures, oligonucleotide primers which would permit PCR amplification and subsequent insertion into the pET bacterial expression vector of S4 cDNA encoding the C-terminal portion of the σ3 were designed.

V8 protease cleaves the σ3 protein between residues 217 (Glu-217) and 218 (Trp-218) (46). The S4 cDNA inserted into the pGEM4 vector (6) was used as the template in PCRs with primers designed to hybridize within the C-terminal region of the S4 open reading frame to yield a product corresponding to amino acids 184 to 365 of σ3. PCR amplification resulted in the deletion of the 5′-terminal 581 nt of the S4 cDNA which includes the open reading frame encoding amino acids 1 to 183 of σ3. An *Nde*I restriction site was introduced into the DNA sequence at the methionine residue 184 of σ3. When inserted into the pET3 vector, the *Nde*I site directed the translational start to a specific site within the S4 open reading frame (Met-184); the σ3 product expressed from pET3-ΔS4 (see Fig. 7) did not include foreign amino acids fused to the N terminus (52).

E. coli cells containing the pET3-ΔS4 construct were induced, and both whole-cell and insoluble protein fractions were examined on SDS-polyacrylamide gels. The induced protein encoded by pET3-ΔS4, designated σ3(184-365), represents amino acids 184 to 365 of σ3. Upon examination of dsRNA-binding activity by the Northwestern blot assay, this N-terminally truncated σ3 protein bound dsRNA (Fig. 2A). Proteolytic digestion of σ3(184-365) with V8 protease resulted in the formation of the σ3 fragment, designated σ3(218-365), which retained dsRNA-binding activity. In addition, some smaller fragments derived from V8-digested virion σ3 (Fig. 1) and recombinant σ3(184-365) (Fig. 2) retained dsRNA-binding activity. The virion-derived and bacterially produced proteins displayed similar proteolytic cleavage patterns, and both tended to multimerize in denaturing SDS-polyacrylamide gels (Fig. 1 and 2).

Because cleavage of σ3(184-365) with V8 protease was necessary to obtain maximal dsRNA-binding activity, a second N-terminal deletion mutant, pET3-V8S4, was constructed to produce a bacterially expressed homolog to the V8 protease-cleaved σ3 protein. The pET3-V8S4 construct encoded a protein which initiated at methionine residue 221 of the σ3 protein sequence, 4 amino acids C terminal to the normal V8 cleavage site. The truncated product encoded by pET3-V8S4 was designated σ3(221-365) (see Fig. 7). When *E. coli* BL21(DE3) was transformed with pET3-ΔS4 or pET3-V8S4 and induced, the sigma 3-derived proteins represented a major fraction of the total protein as measured by staining with Coomassie brilliant blue (Fig. 3, lanes 5 and 8). They were enriched in the insoluble fraction (Fig. 3, lanes 6 and 9). The bacterially produced σ3(221-365) protein encoded by pET3-V8S4 bound dsRNA with an efficiency comparable to that of the V8 protease-generated C-terminal σ3 fragment derived from full-length virion σ3 or recombi-

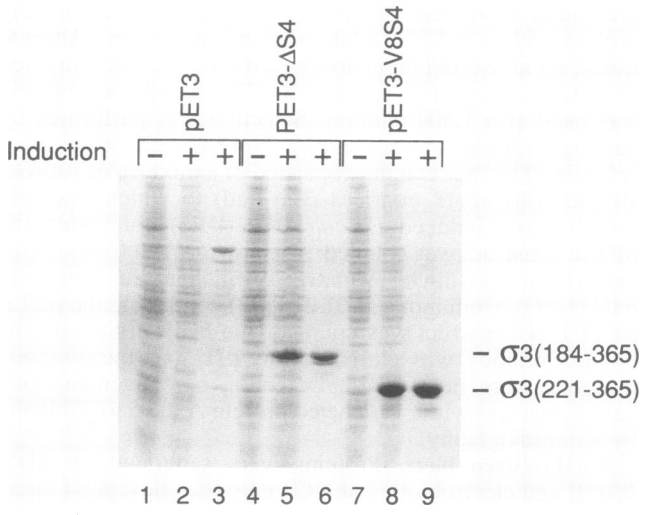

FIG. 3. Expression of N-terminal truncations of σ3 in *E. coli*. Equal portions of total protein (lanes 1, 2, 4, 5, 7, and 8) or insoluble protein (lanes 3, 6, and 9) from uninduced (−) and induced (+) *E. coli* BL21(DE3) transformed with the pET3c vector, the pET3-ΔS4 construct, or the pET3-V8S4 construct were fractionated on a 15% acrylamide gel and stained with Coomassie brilliant blue. Conditions were as described in Materials and Methods.

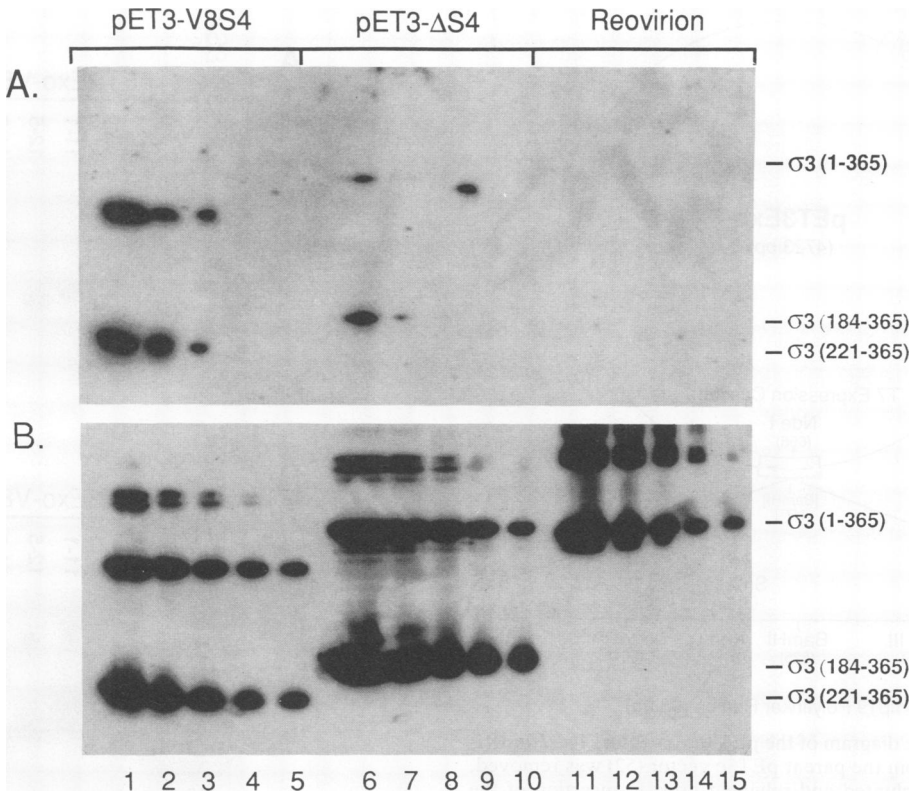

FIG. 4. The N-terminal portion of σ3 interferes with dsRNA-binding activity. Serial twofold dilutions of bacterially produced σ3(184-365) encoded by pET3-V8S4, σ3(221-365) encoded by pET3-ΔS4 protein, or σ3(1-365) protein prepared from reovirions were fractionated on a 15% acrylamide gel and electroblotted to nitrocellulose. (A) Northwestern blot analysis. Lanes 1, 6, and 11, equivalent molar concentrations of protein; lanes 2, 7, and 12, 1/2 the concentrations of protein in lanes 1, 6, and 11, respectively; lanes 3, 8, and 13, 1/4 of the original concentration; lanes 4, 9, and 14, 1/8 of the original concentration; lanes 5, 10, and 15, 1/16 of the original concentration. (B) Western blot of the same filter using polyclonal σ3 antisera. Conditions were as described in Materials and Methods.

nant σ3(184-365) (34). Protein stocks of virion σ3 and the truncated σ3 proteins expressed from pET3-ΔS4 and pET3-V8S4 were normalized for molar protein concentration, and serial dilutions were examined for dsRNA-binding activity by means of a Northwestern blot assay (Fig. 4). The pET3-V8S4-derived σ3(221-365) protein displayed a binding efficiency approximately five times greater than that of the pET3-ΔS4 derived σ3(184-365) protein, as determined by Northwestern blot signal as a function of protein concentration. By contrast, the full-length virion-derived σ3(1-365) protein failed to give a detectable Northwestern signal in the absence of digestion with V8 protease (Fig. 4; compare lanes 2, 7, and 12).

Localization of RNA-binding domain of σ3 to an 85-amino-acid C-terminal region by ExoIII deletion analysis. To further define the region responsible for dsRNA binding, deletion analysis from the C-terminal end of the σ3 protein was performed. Because the commercially available pET expression vectors all contained a single *Bam*HI restriction site in the area of transcriptional termination (52) and otherwise lacked restriction sites which leave 3′ overhangs to protect the flanking vector sequences from ExoIII nuclease treatment, the modified vector pET3Exo was prepared. The pET3Exo vector (Fig. 5), derived from pET3c, contains the polylinker from M13mp19 inserted into the parent pET3c vector at the *Bam*HI site (nt 510), which was destroyed in the process. The inserted polylinker contains *Sac*I and *Kpn*I sites at the 3′ end of the expression cassette, and the polylinker is flanked by primer sites suitable for rapid sequence analysis. In addition, redundant *Eco*RI and *Hin*dIII restriction sites outside of the polylinker were eliminated.

The S4 insert from pET3-V8S4 encoding σ3(221-365) was placed into the pET3Exo vector, and a family of ExoIII deletions was generated. Northwestern blot analysis of ordered, nested deletions clearly defined the C-terminal portion of the σ3 protein (amino acids 221 to 305) as necessary and sufficient for dsRNA-binding activity (Fig. 6A). The amino acid sequence from 283 to 305 was required for binding activity. Mutant 12-5, which terminates with σ3 amino acid residue 305, bound to dsRNA in the Northwestern blot assay, whereas mutant 11-2, which terminates with σ3 amino acid residue 282, did not bind (Fig. 6A and 7). Western blot analysis, performed with the same filters used for the Northwestern blot after removal of the RNA probe, revealed that comparable amounts of σ3-derived protein were loaded in all lanes (Fig. 6B). Furthermore, the Western blot also indicated that the deletion mutant proteins likely multimerize, even when fractionated with a denaturing SDS-polyacrylamide gel.

Features of the dsRNA-binding region of σ3. The dsRNA-binding activity of the 365-amino-acid σ3 protein was localized to the 85-amino-acid region of σ3 encoded by the pET3Exo-V8S4 mutant 12-5, as shown by the Fig. 7 schematic. This region includes a repeated motif of basic amino acids (residues 234 to 240 and 291 to 297). The 23-amino-acid region of the σ3 protein present in mutant 12-5, which bound

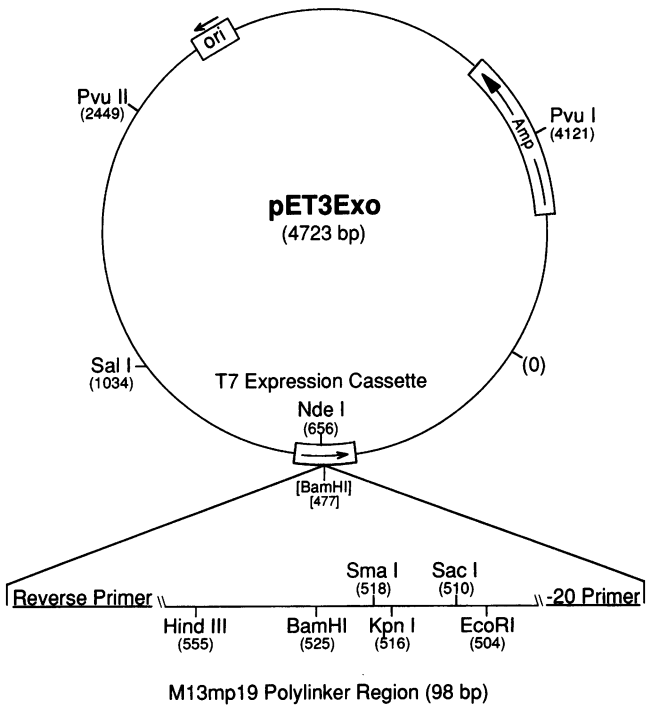

FIG. 5. Schematic diagram of the pET3Exo vector. The *Hin*dIII-to-*Eco*RI segment from the parent pET3c vector (52) was removed, and the vector was blunted and religated prior to insertion of the M13mp19 polylinker region at the blunted *Bam*HI site (originally at nt 477 in the pET3c vector). All sites listed are unique sites. The polylinker region is flanked by the primer sites listed.

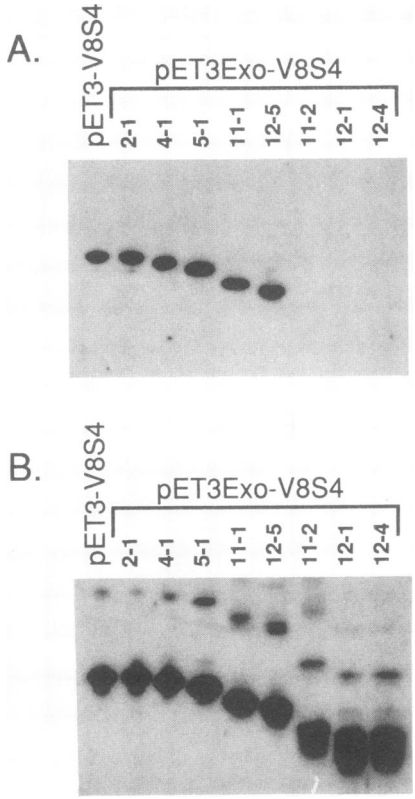

FIG. 6. Amino acids 282 to 305 of the σ3 protein are required for the dsRNA-binding activity. (A) Northwestern blot analysis of insoluble protein produced in *E. coli* BL21(DE3) from the pET3-V8S4 construct or C-terminal deletion mutants derived from the ExoIII-treated pET3Exo-V8S4 constructs (designated 2-1, 4-1, 5-1, 11-1, 12-5, 11-2, 12-1, and 12-4) following denaturing electrophoresis on a 20% acrylamide gel and transfer to nitrocellulose. (B) Western blot analysis of the same filter with immunoselected σ3 antisera followed by ^{125}I-labeled protein A. Conditions were as described in Materials and Methods.

dsRNA, but absent in the mutant 11-2, which did not bind dsRNA, contained the second copy of this basic amino acid motif (amino acids 291 to 297). The region of the σ3 protein from 283 to 305 was predicted to be an α-helix by both the Chou-Fasman (11) and Garnier-Osguthorpe-Robson (19) algorithms. The helical-wheel projection analysis (47) placed the basic amino acids predominantly on one side of an amphipathic helix. The number and position of the basic amino acids within the repeated motif were conserved in the σ3 proteins from all three serotypes of the virus.

The 85-amino-acid region of the σ3 protein which encompassed the dsRNA-binding domain(s) included an area with a high degree of similarity (71% similarity and 39% identity over 28 amino acids) with the 65-kDa regulatory subunit of PP2A (Fig. 8). This region of similarity between σ3 (3) and the regulatory subunit of PP2A (21) was located between the two copies of the basic motif in σ3.

DISCUSSION

The results reported herein demonstrate that recombinant reovirus σ3 protein expressed in *E. coli* displays a V8 proteolytic cleavage pattern and dsRNA-binding activity similar to those of reovirion-derived σ3. The region of the reovirus sigma 3 protein responsible for binding dsRNA was localized by deletion analysis to amino acid residues 221 through 305. This 85-amino-acid region of σ3 includes two copies of a basic amino acid motif separated by a sequence possessing striking sequence similarity to a region of a regulatory subunit of PP2A. These results may be related to several reports in the literature concerning the structure and function of the reovirus σ3 protein and other RNA-binding proteins.

Cleavage of σ3 with V8 protease results in an increase in the dsRNA-binding activity which is associated with the C-terminal fragment. For example, we find in agreement with Schiff et al. (46) that the C-terminal fragment of virion-derived σ3 obtained by V8 protease cleavage possesses dsRNA-binding activity, as measured by a Northwestern blot assay, at both pH 6.5 and pH 7.0. However, the full-length virion-derived σ3 protein from either serotype 1 (Lang) or serotype 3 (Dearing) reovirus did not show dsRNA-binding activity at pH 7.0 as measured by Northwestern blot assay. Although a reduced but clearly detectable dsRNA-binding activity has been described for two full-length reovirus proteins, σ3 (45) and σ2 (14), as measured by Northwestern blot assays performed at pH 6.4 rather than pH 7.0, we find that fragments derived from two *Drosophila* proteins not known to possess RNA-binding activity, Cf1a (amino acids 41 to 290) and pdm-2 (amino acids 57 to 205) expressed by using the pET3 expression system (5), showed dsRNA-binding activity in the Northwestern blot assays performed at or below pH 6.5 (34).

Our findings, together with the previously reported data (see Fig. 5 of reference 46), suggest that the full-length σ3

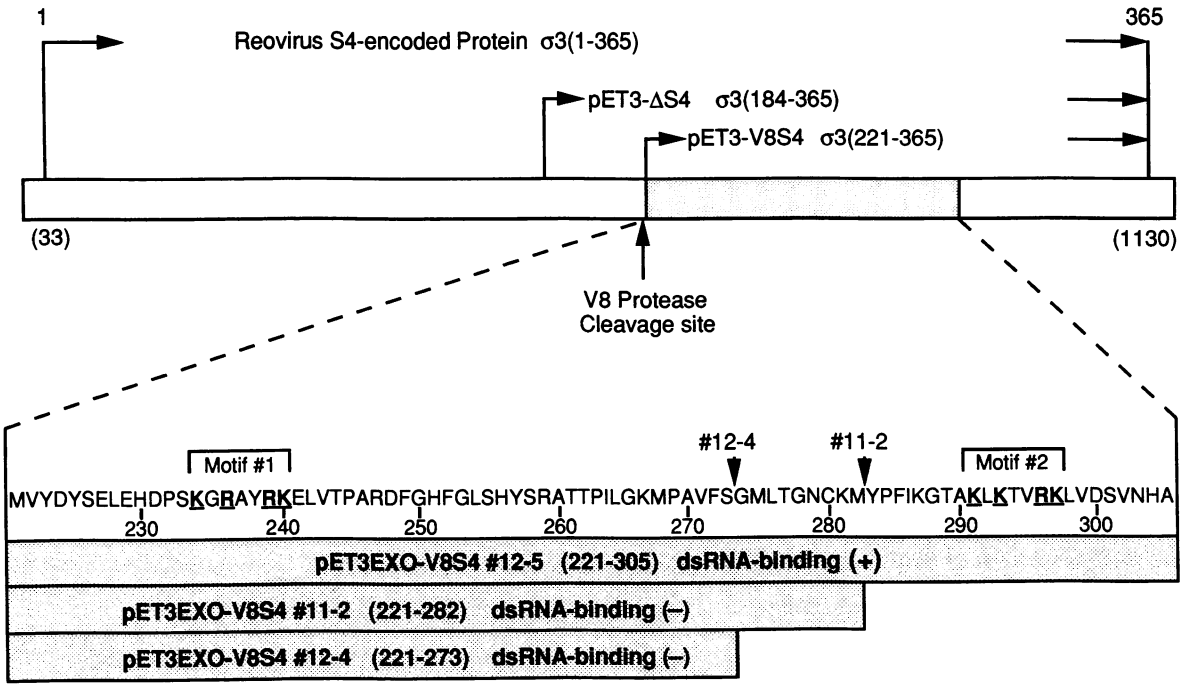

FIG. 7. Schematic summary of the reovirus σ3 deletion mutants and their associated dsRNA-binding activities. The numbers refer to the amino acid sequence present in the σ3 deletion mutant. The basic amino acids which constitute the two candidate binding motifs are underlined.

protein possesses a greatly reduced dsRNA-binding activity at physiological pH relative to the activity of the C-terminal region of σ3. Removal of the N-terminal portion of the σ3 protein, either by use of expression vectors which yield engineered σ3 truncations or by V8 protease treatment to generate σ3 fragments, resulted in an increase in the dsRNA-binding activity of the C-terminal region of σ3. Furthermore, comparison of RNA-binding efficiencies by Northwestern blot analyses of recombinant σ3(184-365) and σ3(221-365) proteins (Fig. 4 and 7) suggests that even the presence of σ3 amino acids 184 to 220 may modulate the binding activity displayed by σ3(221-365). These results imply that the N-terminal region of the σ3 protein attenuates dsRNA-binding activity and suggest a regulatory role during the course of reovirus infection. However, it is conceivable that binding determinants present in the native σ3 protein may be destroyed by incomplete renaturation of the protein during electrophoretic transfer and are not recognized in the Northwestern blotting assay. Similar to our results obtained for reovirus σ3, it has been observed that proteolytically produced C-terminal fragments of the human immunodeficiency virus Tat protein efficiently bind to dsRNA (60), whereas a large excess of the full-length Tat protein is required to observe RNA-binding activity (16).

V8 protease cleavage of virion-derived σ3 occurs primarily at only one of a number of potential cleavage sites (46). Recombinant σ3 appears to be cleaved at the same or an immediately adjacent site, because virion-derived σ3 and recombinant σ3 fragments display similar mobilities on SDS-polyacrylamide gels following digestion with V8 protease (34). This selective cleavage is indicative of an enhanced accessibility of the region to proteases, indicating that this portion of the σ3 protein may be quite flexible. Conceivably the V8 site at glutamic acid 217 may reflect a region of σ3 naturally cleaved by cellular or viral protease during the course of infection. Indeed, preliminary results indicate that infection of human U cells with reovirus serotype 1 resulted in the production of C-terminal proteolytic fragments of σ3 of a size similar to those produced by V8 cleavage (34).

σ3 Protein (240-267) KELVTPARDFGHFGLSHYSRATTPILGK
KELVI AII I ILI II IPILGK
PP2A 65-KDa Subunit (331-358) KELVSDANQHVKSALASVIMGLSPILGK

FIG. 8. Region of similarity between the PP2A 65-kDa regulatory subunit and the reovirus σ3 protein. The middle line denotes the identical or highly conserved (vertical lines) residues.

Repeated basic amino acid motif within the C-terminal region of σ3. The region of σ3 responsible for the dsRNA-binding activity was localized by deletion analysis to amino acid residues 221 to 305. This 85-amino-acid region of σ3 includes two copies of a basic amino acid motif of the form Lys-X-Lys/Arg-X-X-Arg-Lys. This motif appears at residues 234 to 240 and 291 to 297 within the σ3 protein (Fig. 7). Furthermore, the motif is present in the σ3 protein from each of the three serotypes of reovirus (3, 48). Interestingly, the RNA-binding domain of the human RNA-dependent P1/eIF-2α protein kinase also possesses a repeated motif with conserved basic amino acids (32), although the sequence similarity between the repeated σ3 basic motif and the kinase basic motif is low.

By analogy with other RNA-binding proteins, the basic amino acid motifs identified in the C-terminal region of the σ3 protein are likely to be important for the observed dsRNA-binding activity. Basic amino acids play a major role in the RNA-binding activities of several proteins, for example, the RNA recognition motif of ribonucleoproteins such as ribonucleoprotein A (37), arginine-rich motif proteins (8) such as the lambda N antiterminator (28) or human immunodeficiency virus Tat transactivator (54, 60) proteins, and

basic random-coil proteins such as the cowpea chlorotic mottle virus coat protein which appears to undergo an RNA-mediated conformational change necessary for binding (57, 58). Although the RNA targets of these functionally dissimilar proteins vary considerably, the RNA-binding activities of all of these proteins depend upon the interaction of basic amino acid side chains with the sugar-phosphate backbone of RNA. Site-directed mutagenesis will be required to establish which, if any, of the basic amino acid residues present within the repeated basic motifs of σ3 are both necessary and sufficient for the observed RNA-binding activity. Preliminary evidence suggests that both basic motif 1 (residues 234 to 240) and basic motif 2 (residues 291 to 297) contribute to the dsRNA-binding activity (34).

Both virion-derived and recombinant σ3 proteins multimerize. Both virion-derived and bacterially produced σ3 proteins associated to form multimers, even within denaturing SDS-polyacrylamide gels. Multimers of σ3 which lacked the majority of the N-terminal portion of the σ3 protein retain dsRNA-binding activity. It has not been determined whether dsRNA-binding activity requires multimerization, as is the case with the binding of the Rev protein of human immunodeficiency virus to the Rev-response element RNA (61). However, unlike Rev, the basic RNA-binding and multimerization domains of σ3 are separable. For several of the C-terminal deletion mutants, for example, pET3Exo-V8S4 mutants 11-2, 12-1, and 12-4, the dsRNA-binding activity was abolished yet the ability to form multimers was retained. These observations indicate that the sequences responsible for multimerization of σ3 reside within the region between amino acids 221 and 273.

It is unclear whether either of the basic amino acid motifs present within the 85-amino-acid dsRNA-binding region possesses a sufficient charge density to account for the dsRNA-binding activity. However, σ3 dimerization and intramolecular association of the basic motifs are possible mechanisms which could provide the required structural organization and charge density necessary for functional binding. Indeed, dimerization is a well-established mechanism for the activation of DNA-binding proteins (35).

Similarity between the C-terminal dsRNA-binding region of σ3 and other proteins. Similarity between the 85-amino-acid C-terminal region of σ3 which exhibits dsRNA-binding activity and other RNA-binding proteins was negligible, except for the fact that the repeated motif in σ3 contained several basic amino acids. However, the amino acid sequence extending from position 240 to 267 in σ3 showed high sequence similarity with a region of the 65-kDa regulatory A subunit (21) of PP2A (71% similarity and 39% identity over 28 amino acids). This region of similarity was located between the repeated basic amino acid motifs within the dsRNA-binding 85-amino-acid C-terminal region of the σ3 protein.

PP2A has been purified as the eIF-2α protein phosphatase (13). Curiously, the region of the PP2A regulatory subunit which possesses sequence similarity with σ3 resides directly in the middle of a 39-amino-acid motif which is repeated 15 times in the 65 kDa A regulatory subunit (21). Association of the A subunit with the C subunit has been shown to increase the rate of PP2A-dependent dephosphorylation of eIF-2α (10), the substrate of the P1/eIF-2α protein kinase (43), while decreasing the rate of dephosphorylation of the 40S ribosomal subunit (10). Both of these activities would be expected to increase the rate of protein synthesis. Addition of the 55-kDa B regulatory subunit of PP2A to the AC phosphatase complex attenuates phosphatase activity (26). The B subunit must complex with the A subunit in order to associate with the catalytic C subunit (26); therefore, replacement of the A regulatory subunit with a viral counterpart might be expected to increase the activity of the phosphatase through the preclusion of an association with this regulatory subunit. There is precedent for viral recruitment and regulation of PP2A activity. In cells infected with polyomavirus or simian virus 40 the AC form of PP2A is found associated with viral small and medium tumor antigens (38, 59); this association appears to play an important role in the process of cell transformation by polyomavirus (38).

It is tempting to speculate that the C-terminal region of σ3 associated with dsRNA-binding activity fulfills two roles relevant to protein synthesis in reovirus-infected cells. First, σ3 binds to and sequesters free RNA with double-stranded character present in the infected cell, directly interfering with the activation of the RNA-dependent P1/eIF2α kinase (24, 44). Second, σ3 or C-terminal fragments of σ3 may bind to and modulate the activity of the catalytic subunit of PP2A, targeting the virus-regulated phosphatase activity to the area of the PP2A substrate, eIF-2α. This would be consistent with evidence that σ3 protein is involved in the regulation of translation during the later stages of reovirus infection (29) and would represent an efficient mechanism to counter the effects of the interferon-induced, RNA-dependent P1/eIF2α kinase (44).

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