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Regulated, Stable Expression and Nuclear Presence of Reovirus Double-Stranded RNA-Binding Protein σ3 in HeLa Cells

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Reovirus genome segment S4 codes for polypeptide σ3, a major outer capsid component of virions and a double-stranded RNA (dsRNA)-binding protein implicated in viral cytopathogenesis. We have constructed a stable HeLa cell line (S4tTA) that produces functional σ3 under tetracycline transactivator control. In the absence of tetracycline, S4tTA cells synthesized stable dsRNA-binding σ3 that accumulated in the nucleus as well as in the cytoplasm. However, in induced S4tTA cells also expressing reovirus outer shell polypeptide μ1/μ1C, migration of σ3 into the nucleus was blocked, probably as a result of formation of a complex with μ1/μ1C which was exclusively in the cytoplasm. Mutant analyses indicated a correlation between dsRNA-binding activity and nuclear entry of σ3, suggesting an additional role(s) for this capsid protein in virus-cell interactions.

Reovirus genome segment S4 codes for σ3, a pleiotropic polypeptide that binds to double-stranded RNA (dsRNA) (11) and associates with polypeptide μ1 and its cleavage product μ1C to form the outer shell of the mature virion (29). In addition to its important structural role, σ3 has long been implicated in viral cytopathogenesis. For example, genetic reassortment studies suggested that σ3 mediates inhibition of host protein synthesis in infected cells (28) and is also involved in the establishment of viral persistence (1). However, σ3 expressed transiently with reporter chloramphenicol acetyltransferase plasmid in transfected COS-1 cells in the absence of other reovirus proteins enhanced rather than inhibited translation of chloramphenicol acetyltransferase mRNA (9, 26). This stimulation was consistent with the dsRNA-binding activity of σ3, a property which could prevent activation of the dsRNA-dependent protein kinase (PKR) that inhibits translation initiation (12, 19). In agreement with this possibility, σ3 partially restored adenovirus protein synthesis in 293 cells infected with the VAI (virus-associated I) RNA-deficient *dl*331 mutant and also stimulated reporter dihydrofolate reductase synthesis in a VAI RNA-dependent expression system (15). Transient expression of σ3 in HeLa cells also rescued replication of a vaccinia virus mutant lacking *E3L* which codes for an essential, dsRNA-binding protein (2).

The dsRNA-binding activity of σ3 is contained within a C-terminal, 16-kDa fragment that can be separated from the N-terminal Zn-binding fragment by V8 protease cleavage (25). Site-directed mutagenesis and deletion mutant analyses further localized dsRNA binding to a σ3 domain of ~85 residues including several essential basic amino acids (7, 21). These same residues were shown to be necessary for rescue of the *E3L* deletion mutant of vaccinia virus (2), consistent with the hypothesis that these viral dsRNA-binding proteins sequester RNA activators of PKR, sparing host cell initiation capacity for viral mRNA translation.

Recently, a conserved dsRNA-binding domain (dsRBD) of ~70 amino acids has been described for a variety of proteins with diverse functions (20, 30). The nuclear magnetic resonance structures of the dsRBDs from *Escherichia coli* RNase III (14) and *Drosophila* staufen protein (4) were found to consist of a similar α-β-β-β-α topology in which the α-helices are present on one face of a three-stranded antiparallel β-sheet. This arrangement facilitates the electrostatic interaction of key basic residues with the negatively charged surface of duplex RNA. We noticed that the ~85-amino-acid dsRBD of σ3 is also circumscribed by two putative α-helices containing several basic residues necessary for dsRNA binding (7). This implies that σ3 may bind dsRNA by a similar mechanism.

It is well documented that reovirus replication occurs in granular inclusion bodies that develop in the cytoplasm during infection, without obvious nuclear involvement (27). Vaccinia virus replication is also cytoplasmic (22). However, it was recently shown that the vaccinia virus dsRNA-binding E3L protein is present mainly in the nuclei of infected and transfected cells (5, 33). To study further the properties and subcellular localization of reovirus σ3, we have generated a stable HeLa cell line that expresses σ3 under the inducible control of a tetracycline transactivator (tTA) protein (10). Like vaccinia virus E3L protein, σ3 was present in the nucleus and cytoplasm in induced S4tTA cells, suggesting that these dsRNA-binding proteins may function as nuclear factors as well as inhibitors of PKR activation.

MATERIALS AND METHODS

Cells and virus. A HeLa cell line expressing tTA, the tetracycline-controlled transactivator (10), was maintained in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal calf serum and G418 (125 μg/ml). COS-1 cells and reovirus type 3 Dearing strain were obtained from the American Type Culture Collection and grown as described previously (31).

Plasmids. Reovirus type 3 S4 cDNA was subcloned from pBC12BI (9) into the 5′ *Eco*RI and 3′ *Bam*HI sites of pUHD10-3 (10), downstream of the tetracycline (TET) control elements and cytomegalovirus minimal promoter, to obtain S4-pUHD10-3. Reovirus type 3 M2 gene in pBR322 was amplified by PCR with *Xba*I sites engineered at both ends, and the purified product was cloned into the *Xba*I sites of pUHD10-3. The resulting M2-pUHD10-3 was used for transfection of HeLa tTA cells. pHMR272 containing the hygromycin B resistance gene has been described elsewhere (3). The following plasmids were used for transfection of COS-1 cells: pBC12BI containing the type 3 S4 gene under Rous sarcoma virus long terminal repeat control (9), S4 mutants R239I and K291T in pMTVa-(7), and S4 Zn finger motif mutant C51S,C54S (16, 17) subcloned from pBC12BI into the 5′ *Hin*dIII and 3′ *Bam*HI sites of pcDNA3 (Invitrogen).

Stable and transient transfections. To obtain a stable HeLa cell line express-

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ing TET-regulated σ3 (HeLa S4tTA), tTA cells were cotransfected with S4-pUHD10-3 and pHMR272 by calcium phosphate precipitation. Hygromycin B-resistant colonies were isolated after 12 days in selection medium containing 225 U of hygromycin B per ml and 1.5 μg of TET per ml. A clone inducible for σ3 expression upon removal of TET was selected for study and maintained in medium with hygromycin B, G418, and TET. For μ1 expression, HeLa tTA and S4tTA cells were transiently transfected with M2-pUHD10-3 by calcium phosphate precipitation. COS-1 cells were transiently transfected by the DEAE-dextran procedure (31).

Radiolabeling, immunoprecipitation, and Western immunoblotting. Cells were labeled at 48 h posttransfection. Cultures were incubated for 1 h at 37°C in Met-free DMEM, labeled in Met-free DMEM containing 150 μCi of [^{35}S]Met (1,000 Ci/mmol; Amersham) for 20 min, and chased in DMEM for the indicated times. Cells were washed with phosphate-buffered saline (PBS) and lysed by freeze-thawing in hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 0.2 mM magnesium acetate, 0.1% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride). Samples were immunoprecipitated overnight at 4°C or for 2 h at 25°C with a 1:20 dilution of rabbit anti-σ3 antiserum in RIPA buffer (15), and then a 50% slurry of protein A-Sepharose (Pharmacia) was added for 1 h. Precipitates were washed three times with RIPA buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. For Western immunoblot analysis, proteins were separated by SDS–10% PAGE, transferred onto nitrocellulose membranes (Schleicher and Schuell), and assayed by the enhanced chemiluminescence procedure (Amersham). The primary antibody solution contained a 1:1,000 dilution of rabbit anti-σ3 antiserum or a 1:300 dilution of monoclonal antibody to human lactate dehydrogenase H isozyme (Sigma).

Immunofluorescence staining. Cells were seeded into 16-well chamber slides (Nunc, Inc.) at least 24 h prior to fixation (6) and then incubated for 2 h with rabbit anti-σ3 antiserum (1:500 dilution) and/or mouse anti-μ1 monoclonal antibody 10G10 (32) (1:300 dilution). The secondary antibodies, rhodamine-conjugated goat anti-rabbit immunoglobulin G (Calbiochem, 1:100 dilution), and/or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim, 1:100 dilution) were added for 1 h before mounting in aqua-polymount (Polyscience). Staining of σ3 and μ1/μ1C was visualized by red and green fluorescence, respectively.

Cell fractionation. Cells were incubated in hypotonic buffer (see above) at 4°C for 15 min and broken in a Dounce glass homogenizer. Lysates were centrifuged at 1,000 × *g*, and the supernatants were saved as cytoplasmic fractions. The pellets were washed three times with PBS; resuspended in buffer containing 20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride on ice for 30 min; and centrifuged at 12,000 × *g* to obtain the soluble nuclear fractions. Equivalent amounts of cytoplasmic and nuclear fractions were analyzed by SDS-PAGE and Western immunoblotting.

dsRNA-binding assay. σ3 binding to poly(I) · poly(C)-Sepharose beads was assayed as described previously (7).

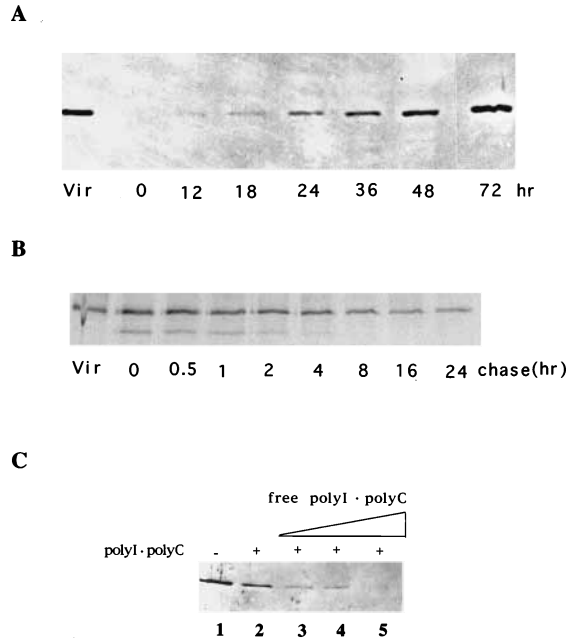

FIG. 1. Induced synthesis and dsRNA binding of σ3. (A) HeLa S4tTA cells were collected at the indicated times after TET removal, and total cell extracts were analyzed by Western immunoblotting with anti-σ3 antibody by using purified reovirus (Vir) as a marker. (B) At 72 h after removal of TET, HeLa S4tTA cells were radiolabeled with [^{35}S]Met and chased as described in Materials and Methods. Extracts prepared at the end of the labeling period (0) and at the indicated times after chase were analyzed by immunoprecipitation with anti-σ3 antibody, SDS-PAGE, and autoradiography. The lane marked Vir contains [^{35}S]Met-labeled reovirus-infected L-cell extract. (C) Samples (20 μl) of an extract of HeLa S4tTA cells (~2 × 10^7 to 5 × 10^7 cells per ml) grown in the absence of TET for 72 h were incubated alone (−) (lane 1) or with poly(I) · poly(C)-Sepharose beads (20 μg of nucleic acid) (+) (lane 2) and 60 (lane 3), 200 (lane 4), or 400 (lane 5) μg of poly(I) · poly(C) as competitor. The beads were then analyzed for bound σ3 by Western immunoblotting.

RESULTS

Induced synthesis of functional σ3 in HeLa S4tTA cells. HeLa tTA cells express an endogenous *E. coli* Tn*10* TET repressor fused to the transactivation domain of herpes simplex virus VP16 protein (10), and the hybrid molecule acts as a transcriptional activator only in the absence of TET. Clonal HeLa S4tTA cells, generated as described above, were examined for σ3 induction in response to TET removal. As shown in Fig. 1A, σ3 was not detected in the presence of TET, indicating effective control by the antibiotic. Within 12 h after TET withdrawal, σ3 was evident and accumulated for at least 72 h. In these cells, σ3 had a half-life of ~8 h by pulse-chase analysis with [^{35}S]Met (Fig. 1B). The σ3 produced in induced S4tTA cells apparently was functional, e.g., about half bound specifically to poly(I) · poly(C)-Sepharose beads under these conditions (Fig. 1C). In addition, σ3 enhanced luciferase expression by five to sixfold in induced S4tTA cells transiently transfected with a cytomegalovirus-driven reporter DNA (data not shown), consistent with the previously described translational effect of transiently expressed σ3 (9, 15, 26).

Subcellular localization of σ3 in HeLa S4tTA cells. A major outer capsid polypeptide like σ3 might be expected to localize in the cytoplasm where reovirus replication and assembly occur (27). However, the dsRNA-binding E3L protein essential for vaccinia virus replication was shown to be one of the few, if not the only, vaccinia virus polypeptide present in the nucleus and cytoplasm in infected and transfected cells (5, 33). In addition, human TAR-binding protein, another dsRNA-binding protein, was found mainly in the nucleus (24). Immunofluorescence staining of HeLa S4tTA cells 48 h after TET removal clearly demonstrated the presence of σ3 in both nuclei and cytoplasm, with greater staining intensity in nuclei (Fig. 2A). Cell fractionation confirmed the presence of nuclear (~63%) and cytoplasmic (~37%) σ3. This distribution contrasts with lactate dehydrogenase (23), which was mainly cytoplasmic (>90%) and served as a control for contamination of the nuclear extracts by cytoplasmic proteins (Fig. 2B).

Nuclear entry of σ3 restricted by coexpression of μ1/μ1C. Since σ3 forms complexes with μ1C and to a lesser extent with μ1 (13, 31), it was of interest to test the effects of reovirus M2 gene expression on the subcellular localization of σ3. In M2-transfected HeLa tTA cells, i.e., in the absence of σ3 expression, μ1/μ1C was exclusively cytoplasmic (Fig. 3A). Staining was in discrete particles, suggesting that μ1/μ1C might be restricted by association with membrane element(s) (31). We next transiently transfected HeLa S4tTA cells with the tTA-regulated M2 expression vector in the absence of TET to induce both σ3 and μ1/μ1C synthesis. Double staining for σ3 and μ1/μ1C demonstrated σ3 exclusively in the cytoplasm of cells that coexpressed μ1/μ1C (compare Fig. 3B and C). By contrast, cells that produced σ3 alone showed nuclear and cytoplasmic staining (Fig. 3B; also Fig. 2A). Similar colocalization of σ3 and μ1/μ1C in the cytoplasm was seen in COS-1 cells transiently cotransfected with Rous sarcoma virus long

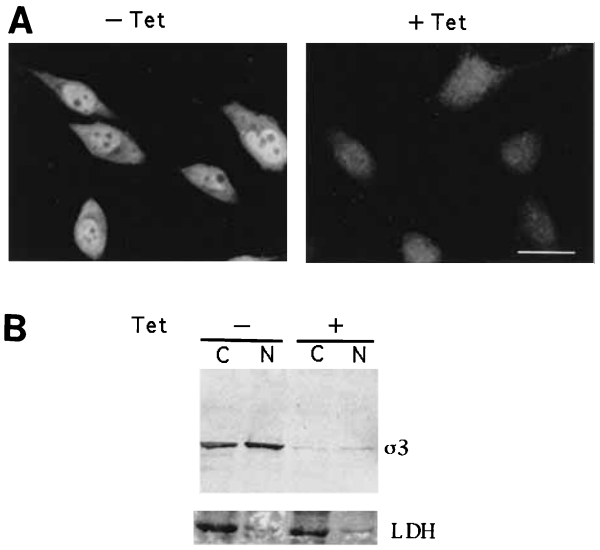

FIG. 2. σ3 in nuclei of induced HeLa S4tTA cells. (A) Cells grown in medium without TET (−Tet) or with TET (+Tet) were analyzed for σ3 by immunofluorescence staining. Bar, 100 μm. (B) Cells were fractionated as described in Materials and Methods, and cytoplasm (C) and nuclei (N) were sequentially analyzed for σ3 (top) and lactate dehydrogenase (LDH) (bottom) by Western immunoblotting followed by densitometry.

terminal repeat-driven M2 and S4 expression vectors (data not shown). The results indicate that the subcellular location of σ3 is changed by coexpression of μ1/μ1C, suggesting that μ1/μ1C may also modulate the function(s) of σ3.

Correlation between the nuclear localization and dsRNA-binding activity of σ3. The Rev protein of human immunodeficiency virus contains a domain that acts as a nuclear localization signal and as an RNA-binding site (18). In addition, the dsRNA-binding activity of the vaccinia virus E3L protein appears to correlate with its presence in the nucleus, since the treatment of cells with RNase abrogated E3L nuclear localization (33). However, a recent report argued against this correlation on the basis of E3L mutant studies (5). Since reovirus σ3 contains a potential nucleic acid-binding Zn finger motif and a separate dsRNA-binding domain (25), we tested which region might be involved in the nuclear localization of σ3.

Site-specific σ3 mutants altered in the dsRNA-binding domain (R239I and K291T) or the Zn finger motif (C51S,C54S) (16) were expressed in COS-1 cells. Indirect immunofluorescence staining demonstrated that wild-type σ3 was present in nuclei and in the cytoplasm of COS-1 cells (Fig. 4A), in agreement with results obtained in induced S4tTA cells. In contrast, the three mutant proteins showed dramatically altered staining patterns despite the fact that they were expressed at the wild-type level (Fig. 4E). In each case, mutant proteins localized to

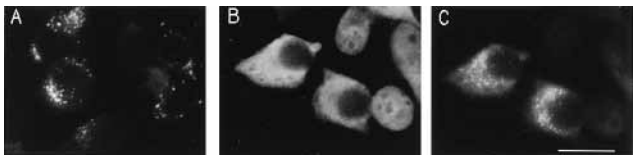

FIG. 3. Effect of coexpressed μ1/μ1C on subcellular localization of σ3. (A) HeLa tTA cells in the absence of TET were transfected with tTA-regulated M2 cDNA and after 72 h were stained for μ1. (B and C) HeLa S4tTA cells were transfected with M2 cDNA as described for panel A, double-stained, and examined separately for σ3 (B) and μ1 (C). Bar, 100 μm.

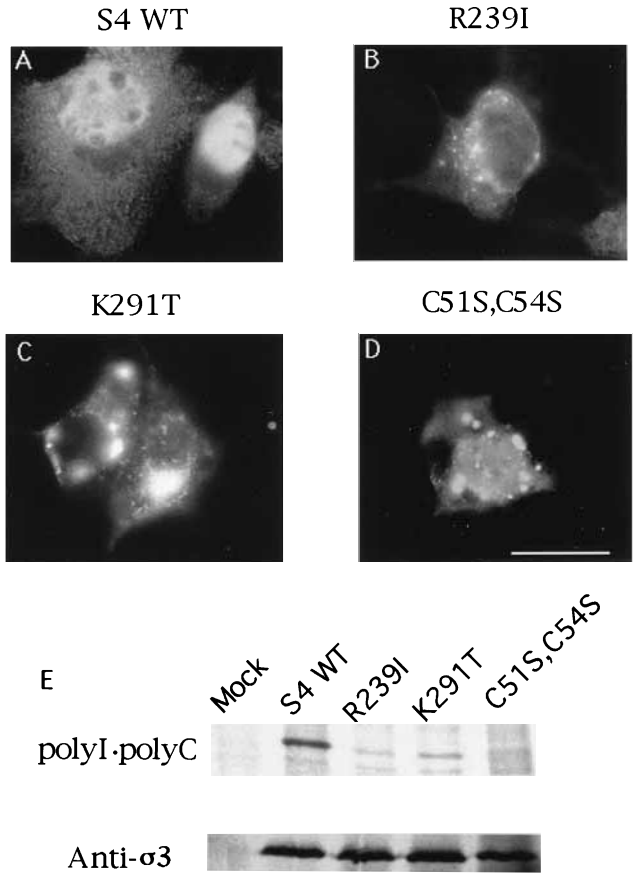

FIG. 4. Subcellular localization of σ3 correlates with dsRNA binding. COS-1 cells were transfected to express wild-type (WT) σ3 (A) and mutants R239I (B), K291T (C), and C51S,C54S (D), and then the cells were subjected to immunofluorescence staining. Bar, 100 μm. (E) Cells transfected with wild-type (WT) or mutant S4 were labeled with [^{35}S]Met. Cell extracts were analyzed for σ3 by poly(I) · poly(C)-Sepharose binding and immunoprecipitation.

brightly staining cytoplasmic granules. For mutants R239I and K291T, staining appeared to be almost exclusively cytoplasmic (Fig. 4B and C), and the dsRNA-binding activity of these mutants was markedly reduced from that of the wild-type σ3 (Fig. 4E). The dsRNA-binding activity of the C51S,C54S mutant was also significantly diminished, but staining in nuclei and cytoplasmic granules was observed (Fig. 4D and E).

A previous study of the C-terminal fragment of σ3 obtained by V8 protease digestion indicated that removal of the Zn finger motif enhanced dsRNA binding (21). This result suggested that the N- and C-terminal regions of σ3 may interact to decrease dsRNA binding. Consistent with this possibility, mutation of the Zn finger motif (C51S,C54S) may alter the structure of the protein in a way that masks the dsRBD, resulting in decreased dsRNA binding. Taken together, these results suggest that the migration of σ3 into the nucleus correlates directly or indirectly with dsRNA binding.

DISCUSSION

Although it has been difficult to establish stable cell lines expressing gene products that interfere with PKR activation, e.g., adenovirus VAI RNA (18a), we have constructed a HeLa cell line that produces reovirus polypeptide σ3 under control of the tTA regulator. The induced σ3 protein was apparently

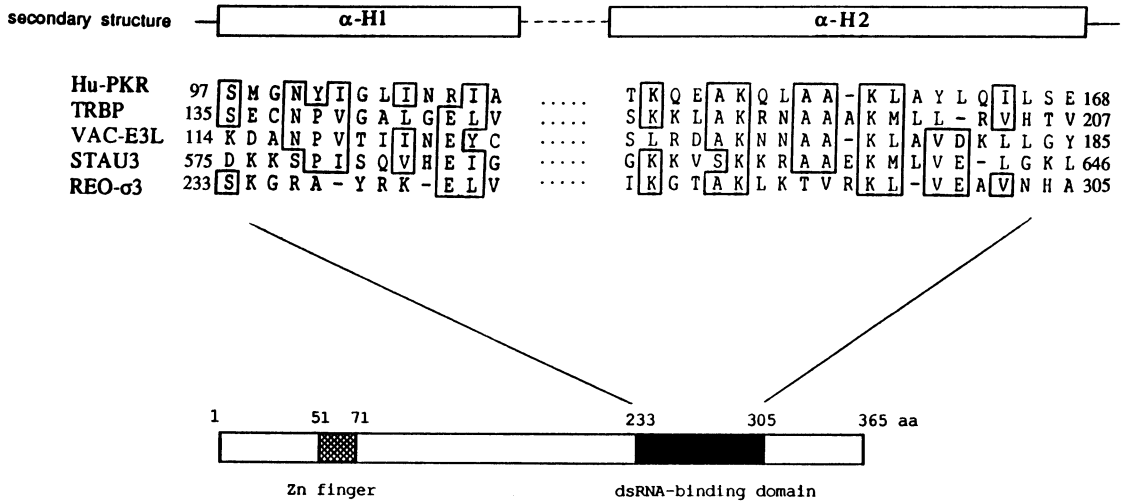

FIG. 5. Schematic of σ3 showing the location of the Zn finger motif and conserved dsRNA-binding domain. The two predicted α-helices (α-H1 and α-H2) from amino acids (aa) 233 to 305 of σ3 (REO-σ3) (accession no. K02739) were aligned with homologous counterparts (amino acids indicated by numbers) in human PKR (Hu-PKR) (accession no. M35665), human TAR-binding protein (TRBP) (accession no. M60801), vaccinia virus E3L (VAC-E3L) (accession no. M36339), and *Drosophila* staufen protein dsRBD3 (STAU3) (accession no. M6911). Conserved amino acids are boxed.

functional, as it retained dsRNA-binding activity (Fig. 1C) and stimulated the expression of a luciferase reporter gene (data not shown). Consistent with an absence of detectable effects of σ3 on host protein synthesis in cells transiently transfected with S4 expression vectors (15), the HeLa S4tTA stable cell line grew equally well in the presence or absence of TET and with a doubling time close to that of the parental HeLa tTA cells (data not shown). These results indicate that σ3 alone is not directly responsible for inhibiting host protein synthesis and/or the accompanying cytopathic effects in reovirus-infected cells (28).

As in infected cells, σ3 induced in S4tTA cells was present in the cytoplasm. In contrast to infected cells, σ3 was also found in the nucleus in S4tTA cells. This appears to be uncharacteristic for a major capsid protein of a cytoplasmic virus. Passive diffusion could account for nuclear entry of σ3 (8). However, the two short stretches of basic residues essential for σ3 dsRNA-binding activity may act as *cis* elements, e.g., nuclear localization signals for active nuclear import. This is supported by the finding that the R239I and K291T mutant proteins affected in each of these basic stretches were retained in the cytoplasm (Fig. 4B and C). Human immunodeficiency virus type 1 Rev protein is a well-documented example where nuclear localization signal function and RNA-binding activity are correlated (18). The σ3 Zn finger motif mutant (C51S,C54S) contains an intact dsRBD, but its presence in the nucleus and dsRNA-binding activity were both decreased (Fig. 4). This may reflect an effect of the mutation on σ3 folding, resulting in cytoplasmic aggregation or other changes that impact negatively on the σ3 dsRBD. These possibilities remain to be explored further.

One of the most characteristic properties of σ3 is its dsRNA-binding activity. The dsRBD has been localized to a sequence of ~85 amino acids in the C-terminal region (21). Within this domain are two separate basic stretches that are predicted to fold as α-helices. These regions can be aligned with sequences that display similar predicted secondary structures in the dsRBDs of several dsRNA-binding proteins, including the *Drosophila* staufen protein (Fig. 5). Three basic residues in this region of staufen have been shown to be involved in dsRNA binding: K-50 (numbered as in reference 4 and aligned with K-287 in σ3, [Fig. 5]), K-51, and K-54 (corresponding to σ3 K-291 [Fig. 5]). On the basis of our results and on the studies of others (7), it is clear that mutation of these basic residues in σ3 resulted in diminished dsRNA-binding activity. It is therefore likely that σ3 includes a conserved dsRBD similar in three-dimensional structure to that of staufen dsRBD3, in spite of the limited sequence homology of σ3 to other dsRNA-binding proteins.

Reovirus replication occurs in cytoplasmic inclusions that form at early stages of infection. As infection progresses, these granules migrate toward the nucleus and increase in size but apparently remain cytoplasmic (27). This is probably due to the association of σ3 with μ1/μ1C (13, 31), blocking the import of the majority of σ3 into the nucleus, as observed in transfected cells (Fig. 3B). At later stages of infection, free σ3 molecules that have not been incorporated into virion outer shells could migrate or diffuse to the nucleus. Immunofluorescence analysis of σ3 localization in infected HeLa cells at later stages of infection revealed strong perinuclear staining but also some staining within nuclei (unpublished results). Reovirus σ3 is an inhibitor of PKR, suggesting that σ3 is involved in translational control (12, 15). How, if at all, the presence of σ3 in the nucleus relates to PKR inhibition is not clear. In studies with mutant vaccinia virus E3L dsRNA-binding proteins, the rescue of E3L-deleted virus correlated with dsRNA-binding activity but not with migration of E3L protein to the nucleus (5). This indicates that cytoplasmic E3L is important for blocking PKR activation. It will be of interest to explore the possibility that σ3 has another role(s) in addition to its dsRNA-binding and capsid-forming functions.

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