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# Translational Stimulation by Reovirus Polypeptide σ3: Substitution for VAI RNA and Inhibition of Phosphorylation of the α Subunit of Eukaryotic Initiation Factor 2

RAMONA M. LLOYD AND AARON J. SHATKIN*

*Center for Advanced Biotechnology and Medicine, 679 Hoes Lane, Piscataway, New Jersey 08854-5638*

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**COS cells transfected with plasmids that activate DAI depend on expression of virus-associated I (VAI) RNA to prevent the inhibitory effects of the α subunit of eukaryotic initiation factor 2 (eIF-2α) kinase (DAI) and restore the translation of vector-derived dihydrofolate reductase mRNA. This VAI RNA requirement could be completely replaced by reovirus polypeptide σ3, consistent with its double-stranded RNA (dsRNA)-binding activity. S4 gene transfection of 293 cells also partially restored adenovirus protein synthesis after infection with the VAI-negative *dl*331 mutant. In *dl*331-infected 293 cells, eIF-2α was present mainly in the acidic, phosphorylated form, and *trans* complementation with polypeptide σ3 or VAI RNA decreased the proportion of eIF-2α(P) from ~85 to ~30%. Activation of DAI by addition of dsRNA to extracts of S4 DNA-transfected COS cells required 10-fold-higher levels of dsRNA than extracts made from cells that were not producing polypeptide σ3. In extracts of reovirus-infected mouse L cells, the concentration of dsRNA needed to activate DAI was dependent on the viral serotype used for the infection. Although the proportion of eIF-2α(P) was greater than that in uninfected cells, most of the factor remained in the unphosphorylated form, even at 16 h after infection, consistent with the partial inhibition of host protein synthesis observed with all three viral serotypes. The results indicate that reovirus polypeptide σ3 participates in the regulation of protein synthesis by modulating DAI and eIF-2α phosphorylation.**

A strategy often used by infected animal cells to block virus propagation involves repression of translation initiation by the inhibitory action of DAI, the interferon-induced, double-stranded (ds) RNA-activated kinase (11, 31). The substrate for this highly specific kinase is the α subunit of eukaryotic initiation factor 2 (eIF-2α). This factor complexed with GTP targets the methionine initiator tRNA to the 40S ribosomal subunit (10). The subsequent joining of the 60S subunit to form 80S initiation complexes is dependent on hydrolysis of the GTP. For continued catalysis of initiation by eIF-2, the resulting GDP must be replaced by GTP in a process that is mediated by a guanine nucleotide exchange factor (29). However, eIF-2-GDP complexes that contain an α subunit phosphorylated by DAI bind to the exchange factor with higher affinity, preventing eIF-2 recycling and resulting in loss of translation initiation (28).

A number of animal viruses have evolved mechanisms to evade the cellular interferon response by eliciting inhibitors of DAI. For example, vaccinia virus codes for a specific kinase inhibitory factor, a dsRNA-binding protein that can prevent DAI activation (1). The vaccinia virus K3L protein also inhibits DAI activation and may act as a kinase decoy in vaccinia virus-infected cells (5) by virtue of its high sequence homology to the region of eIF-2α that is phosphorylated by DAI (3). Influenza virus infection activates a 58-kDa cellular protein that counteracts DAI function (22, 23). In adenovirus-infected cells, virus-associated I (VAI) RNA interacts with DAI to prevent activation by dsRNA (26), and short human immunodeficiency virus transcripts containing the Tat-responsive TAR region may act similarly to inhibit DAI activation and preserve viral protein synthesis (9).

Mammalian reoviruses also express gene products that can influence translation apparently by modulating DAI. The reovirus S4 gene encodes polypeptide σ3, a major outer capsid protein that binds dsRNA (13, 32). Consistent with its dsRNA-binding activity, σ3 stimulates translation of reporter gene mRNA, e.g., chloramphenicol acetyltransferase, during transient coexpression in COS cells (7) and probably corresponds to the DAI inhibitor in extracts of reovirus-infected mouse L cells (15). In contrast to the translation stimulatory activity of σ3 in transfected cells, the S4 gene has been implicated in serotype-specific inhibition of host mRNA translation in reovirus-infected cells (35). These phenotype mapping results support a function for σ3 in translation. However, they also potentially create a paradox, i.e., stimulation of reporter gene expression by σ3 in transfected cells versus S4 gene segregation with host inhibition in infected cells.

To explore the role of the S4 gene in protein synthesis, we analyzed the effects of σ3 on DAI activation, eIF-2α phosphorylation, and translation in transfected cells and in cells infected with reovirus and adenovirus. The results demonstrate that the reovirus S4 gene can substitute for VAI RNA under conditions that require inhibition of DAI in order to facilitate initiation of mRNA translation. They suggest further that protein synthesis is regulated in reovirus-infected cells by the availability of polypeptide σ3 to modulate DAI activation.

## MATERIALS AND METHODS

**Virus and cells.** Reovirus serotype 1 Lang strain, type 2 Jones, and type 3 Dearing as well as mouse L929 cells were obtained from the American Type Culture Collection. Adenovirus type 5 was from Daniel Klessig (Rutgers University), and the VAI-negative adenovirus mutant *dl*331 was provided by Robert Schneider (New York University Med-

* Corresponding author.

ical Center). Monkey kidney COS-7 cells obtained from Bryan Cullen (Duke Medical Center), COS-1 cells from Daniel Klessig, and human embryonic kidney 293 cells from Michael Mathews (Cold Spring Harbor Laboratory) were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). L929 cells were grown in minimal essential medium supplemented with 5% heat-inactivated FBS.

For virus infections, L929 or 293 cells were seeded at $10^6$ cells per 35-mm dish. The medium was aspirated 16 h later and replaced with ~0.1 to 0.5 ml of phosphate-buffered saline (PBS) containing virus at the indicated multiplicity of infection. After virus absorption at 37°C for 2 h, the cells were refed with medium supplemented as appropriate.

**Plasmids and transient transfections.** pBR322-based vectors pD61 and p91023 containing the dihydrofolate reductase (DHFR) gene under control of the adenovirus major late promoter were provided by Randal Kaufman (Genetics Institute). p91023, but not pD61, also carries the adenovirus sequence coding for VAI and VAII RNA. pt3S4, which expresses reovirus type 3 polypeptide σ3 under control of the Rous sarcoma virus long terminal repeat promoter, and pt3S4inv, in which the 5′ two-thirds of the S4 gene is inverted, have been described previously (7). COS cells were transfected by the DEAE-dextran procedure essentially as described previously (18).

Transfection of 293 cells with pMHVA (from M. Mathews [27]) or pCMVS4, prepared by inserting S4 in place of the adenovirus E1B 19-kDa gene in pCMV19K (from Eileen White, Center for Advanced Biotechnology and Medicine), was done by coprecipitation of DNA with calcium phosphate (38) according to the supplier's instructions (GIBCO-BRL transfection system). Briefly, calcium phosphate was added to plasmid DNA mixed with 1× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline. The mixture was added to subconfluent 293 cells in 12-well culture plates with 1.5 ml of DMEM containing 10% FBS. After incubation overnight, the DNA-containing medium was replaced with DMEM and 10% FBS and incubated at 37°C.

**[$^{35}$S]methionine labeling.** Cells were incubated in methionine-free DMEM without serum for 1 h at 37°C at the indicated times after transfection and/or infection. The medium was then replaced with 1 ml of methionine-free DMEM (without FBS) containing 100 μCi of [$^{35}$S]methionine per 35-mm plate (specific activity, 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). After 30 to 60 min, an additional 1 ml of DMEM (with methionine) was added for 15 min as a chase. The medium was removed by aspiration, and the cells were washed with PBS before lysis by incubation for 5 min at room temperature in 0.5 ml of radioimmunoprecipitation assay buffer (RIPA; 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.01 M Tris-HCl [pH 7.4], 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride). Samples transferred to Eppendorf tubes were clarified by centrifugation at 16,000 × *g* for 10 min, and the supernatants were boiled in electrophoresis loading buffer before analysis by SDS-polyacrylamide gel electrophoresis (20).

**DAI phosphorylation.** S10 extracts were prepared from virus-infected or transfected cells (30) and assayed for DAI activation by autophosphorylation. Cell protein (5 μg) and purified reovirus dsRNA as indicated were combined in a 10-μl reaction mixture (20 mM HEPES [pH 7.5], 50 mM KCl, 4 mM $MgCl_2$, 1.5 mM dithiothreitol, 0.1 mM ATP) supplemented with 10 μCi of [γ-$^{32}$P]ATP (specific activity, 3,000 Ci/mmol; Amersham) and incubated for 10 min at 31°C; this was followed by the immediate addition of NaF to 20 mM. The sample was diluted to 100 μl with TENN buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40), and 10 μl of a 10% solution of washed Pansorbin (Calbiochem) was added. After centrifugation to remove the Pansorbin, the supernatant was incubated with 1 μl of a monoclonal antibody directed against an *Escherichia coli*-expressed clone of DAI (kindly provided by T. Shenk, Princeton University). After incubation for 60 min, 30 μl of Pansorbin was added, and 10 min later, the Pansorbin and attached antibody complexes were pelleted (16,000 × *g*, 1 min) and washed five times in TENN before resuspension in buffer for analysis by SDS-PAGE.

**Vertical slab isoelectric focusing.** Cell pellets were resuspended at 3 × $10^6$ cells per ml in PBS, and 40 μl was added directly to 60 mg of urea and 15 μl of 7× sample buffer (1× is 3% ampholines mixed 1:4 [3–10:5–7], 2% β-mercaptoethanol, 0.4% Nonidet P-40). Samples were kept on ice and brought to room temperature 30 min before analysis by vertical slab isoelectric focusing (33). Urea gels containing 5.2% acrylamide, 0.29% bisacrylamide, 3% ampholines mixed 1:4 (3–10:5–7), and 9 M urea (16.5 g in a 30-ml total volume) were prefocused for 15 min at 200 constant volts (V), 30 min at 300 V, and 30 min at 400 V in electrode solutions of degassed 20 mM NaOH (upper) and 10 mM phosphoric acid (lower). The upper buffer was replaced with fresh buffer when the samples were loaded. Electrophoresis was done at 3 W constant power for 16 h and then 800 V for 60 min.

**eIF-2α assay.** Polypeptides in vertical slab isoelectric focusing gels were electrotransferred to an Immobilon P membrane (polyvinylidene difluoride; Millipore) for 60 min at 10 V in 0.7% acetic acid with a Bio-Rad semi-dry transblot apparatus. The blot was then fixed in 0.5% glutaraldehyde in PBS for 30 min, washed three times for 10 min each in TBS (10 mM Tris-HCl [pH 7.5], 0.15 M NaCl), and blocked by incubation for 1 h at 37°C with 3% bovine serum albumin (BSA) in PBS before incubation with a monoclonal antibody to eIF-2α (from Edgar Henshaw, University of Rochester). After incubation with the antibody diluted 1:1,000 in 1% BSA for 1 h, the blot was washed four times for 5 min with PBS containing 0.05% Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad) in 1% BSA for 1 h at room temperature. The blot was subsequently washed four times for 5 min in PBS with 0.05% Tween 20 before detection of the eIF-2α with an enhanced chemiluminescence detection system (Amersham).

## RESULTS

**Replacement of adenovirus VAI by reovirus S4 gene expression.** Transient transfection with the adenovirus VAI gene can stimulate the expression of the reporter gene for DHFR by greater than 10-fold (19). An analogous stimulation of CAT expression has been observed with the reovirus S4 gene (7). The mechanism of action of VAI RNA in restoring translation by preventing the inhibitory effect of DAI has been well documented (26). It was therefore of interest to assess the ability of the reovirus S4 gene to replace VAI in a system in which translation is dependent on DAI inhibition.

Expression of DHFR in COS cells transfected with the VAI-negative pD61 is severely repressed, apparently due to localized activation of DAI by symmetrical plasmid transcription (2, 18). This inhibitory effect was overcome in

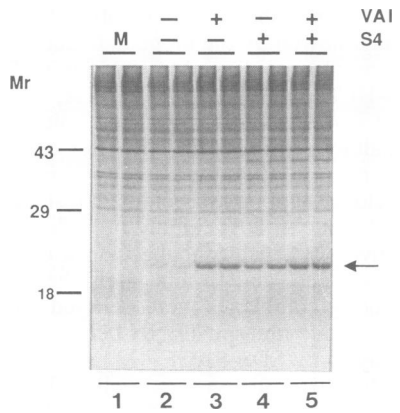


FIG. 1. Replacement of VAI RNA by polypeptide σ3 in a transfection system dependent on DAI inhibition for translation of plasmid-derived DHFR mRNA. COS-1 cells were cotransfected with 1.0 μg of *DHFR*-containing vector p91023 (VAI$^+$) or pD61 (VAI$^-$) and 1.0 μg of either pt3S4 (S4$^+$) or pt3S4inv (S4$^-$). At 48 h after transfection, cells were radiolabeled with [$^{35}$S]methionine, and lysates of duplicate cultures were analyzed by SDS-PAGE and autoradiography. Positions of marker polypeptides are shown on the left, and the arrow indicates the DHFR band. Note also the band of σ3 migrating slightly below the 43-kDa marker in lanes 4 and 5.

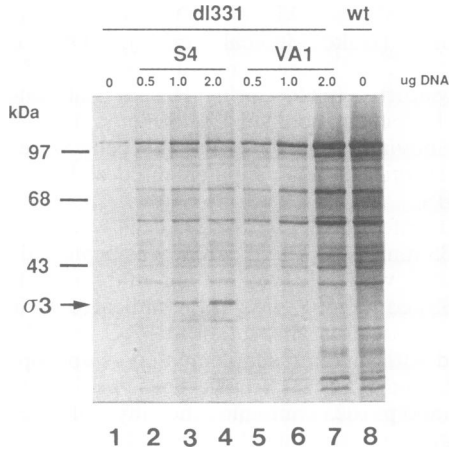


FIG. 2. *trans* complementation of adenovirus protein synthesis by polypeptide σ3 in cells infected with the VAI-negative mutant. Human 293 cells were transfected with the indicated amounts of pCMVS4 (S4) or pMHVA (VAI) and infected 24 h later with 20 PFU of wild-type (wt) adenovirus 5 or the VAI-negative *dl*331 mutant per cell. Cells were labeled with [$^{35}$S]methionine at 20 h after infection, and lysates were analyzed by SDS-PAGE and autoradiography.

p91023-transfected cells in which expression of VAI RNA rescued translation of the plasmid-derived DHFR mRNA. On the basis of its dsRNA-binding activity and enhancement of chloramphenicol acetyltransferase expression, it was predicted that σ3 would replace VAI RNA and restore the translation of DHFR mRNA in pD61-transfected COS cells. Conversely, the presence of the S4 gene was expected to have little or no effect on reporter mRNA translation in p91023-transfected cells. As shown for duplicate samples in Fig. 1, pD61-transfected cells expressed very little DHFR in the absence of the VAI or S4 gene (lane 2). However, when serotype 3 polypeptide σ3 was produced by cotransfection with pt3S4, reporter mRNA translation was markedly increased (Fig. 1, lane 4) to a level similar to that in VAI RNA-producing, p91023-transfected cells (lane 3). Expression of σ3 in pt3S4-p91023 cotransfected cells had little additional effect on DHFR synthesis (Fig. 1, lane 5). Similar results were obtained with plasmids expressing the reovirus type 1 or type 2 polypeptide σ3 (24). In other experiments, DHFR expression from the VAI RNA-independent pMT2 vectors was unaffected by cotransfection with the S4 gene (24), consistent with dsRNA activators not being produced from these pUC18-based vectors (18).

To confirm that the stimulation of the reporter DHFR occurred at the level of mRNA translation, as reported previously for chloramphenicol acetyltransferase stimulation (7), we assayed steady-state levels of transcripts by Northern (RNA) blot analysis with a radiolabeled *Eco*RI fragment of DHFR DNA as probe. The amount of DHFR mRNA in all samples was similar and varied by less than twofold (24).

**S4 complementation of *dl*331.** The adenovirus VAI-negative mutant *dl*331 replicates poorly in 293 cells (36) because of its inability to block the activation of DAI that is mediated by overlapping viral transcripts (25). In *dl*331-infected cells, 80 to 90% of eIF-2α is in the phosphorylated state, resulting in almost complete inhibition of both viral and cellular protein synthesis. VAI RNA produced in *trans* from a transfected plasmid can partially restore translation in adenovirus-infected cells (27).

The finding that the reovirus S4 gene can replace VAI RNA function in transfected COS cells encouraged us to test the effects of S4 gene expression in adenovirus-infected cells. Human 293 cells were transfected with pCMVS4, a vector containing the S4 gene driven by the cytomegalovirus promoter, or with pMHVA, which expresses VAI RNA from the intragenic control region that functions as the polymerase III promoter. Transfected cells were allowed to express the plasmid-encoded genes for 24 h before infection with *dl*331 at a multiplicity of infection of 20 PFU per cell. At 20 h postinfection, the cells were labeled with [$^{35}$S]methionine and analyzed by SDS-PAGE to ascertain the extent of translational restoration by the plasmid expressed genes. The results in Fig. 2 demonstrate that the VAI gene expressed in *trans* restores late viral protein synthesis in a DNA concentration-dependent manner. Translation stimulation by plasmid-derived VAI RNA was almost complete at 2 μg of VAI-expressing plasmid and corresponded to ~80% of the level of late viral protein synthesis in wild-type infections (Fig. 2, lanes 7 and 8). Viral protein synthesis was also partially restored by expression of the transfected S4 gene but notably less well (Fig. 2, lanes 2 to 4). Although σ3 expression increased in parallel with pCMVS4 DNA concentration, the maximum restoration was only ~20% of wild-type-infected cell levels and was achieved at 1 μg of plasmid DNA. This result suggests that σ3 was less effective than VAI RNA in preventing the phosphorylation of eIF-2α in adenovirus-infected 293 cells. It may also reflect the replacement of a (VAI) RNA function by a polypeptide (σ3) under conditions of repressed protein synthesis.

**DAI in reovirus-infected cells.** The presence of DAI in reovirus-infected cells was measured by autophosphorylation of the kinase in extracts prepared at 16 h after infection. The extracts were incubated with [γ-$^{32}$P]ATP, immunoprecipitated with a DAI-specific antibody, and visualized by SDS-PAGE and autoradiography. As shown in Fig. 3, dsRNA-activatable DAI was present in extracts of cells infected with each of the three reovirus serotypes. In other

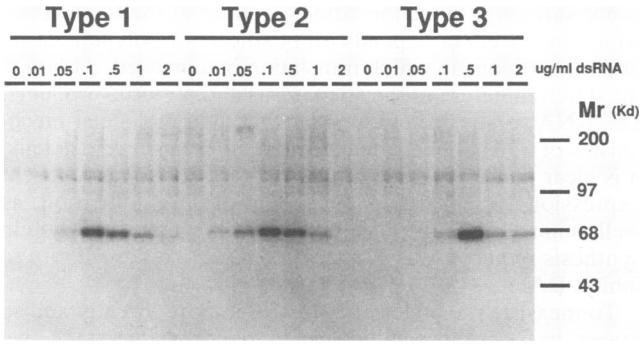


FIG. 3. Activation of DAI by addition of dsRNA to extracts of reovirus-infected mouse L929 cells. Lysates were prepared at 16 h after infection with 10 PFU of the indicated reovirus serotype per cell. DAI activation was assayed by autophosphorylation in lysates incubated with [γ-$^{32}$P]ATP and dsRNA, immunoprecipitation, SDS-PAGE, and autoradiography as described in Materials and Methods.

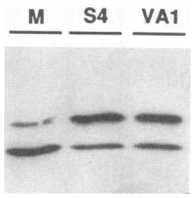


FIG. 5. Decreased phosphorylation of eIF-2α in *dl*331-infected cells transfected with VAI or S4 DNA. Human 293 cells were mock transfected (lane M) or transfected with 5.0 μg of pCMVS4 (lane S4) or pMHVA (lane VAI) and 24 h later were infected with 20 PFU of the adenovirus *dl*331 mutant per cell. S10 extracts prepared 20 h after infection were analyzed for eIF-2α by vertical slab isoelectric focusing and Western blotting (immunoblotting) as described in Materials and Methods. The lower band in the autoradiogram corresponds to eIF-2α(P), the phosphorylated, more acidic form of eIF-2α. The fraction of eIF-2α(P) in the M, S4, and VAI samples as measured by densitometry corresponded to 85, 30, and 30%, respectively.

experiments, extracts of mock-infected L cells were shown to contain at least 10-fold-less activatable DAI, with maximal activation at a dsRNA concentration of 0.01 μg/ml (24). In infected cell extracts, the levels of DAI activation increased in response to addition of dsRNA and characteristically decreased at higher dsRNA levels (14). The minimal amount of exogenous dsRNA needed to detect phosphorylated DAI in extracts of type 2-infected cells was severalfold less than for type 1 and type 3 infections, and maximal phosphorylation required approximately five times more dsRNA in type 3 extracts (Fig. 3). These findings suggest that the amount of σ3 available to bind exogenous dsRNA and prevent DAI activation was less in extracts of type 1- and 2-infected cells than in extracts of type 3-infected cells. However, correlations of σ3 and DAI levels are complicated by the presence in infected cells of other viral products that can also influence DAI activation, as discussed below.

**DAI in S4 gene-transfected cells.** To determine whether the presence of σ3 influences DAI activation in uninfected cells, we also assayed extracts prepared from COS-7 cells transfected with pt3S4 or pt3S4inv for DAI phosphorylation (Fig. 4). Maximal phosphorylation of DAI required ten times more dsRNA in extracts that contained σ3, consistent with its dsRNA-binding activity. Since only 30% of the COS cells were transfected as determined by immunofluorescence with σ3-specific antibody (data not shown), the band corresponding to the phosphorylated kinase was noticeably weaker than in reovirus-infected mouse L cells.

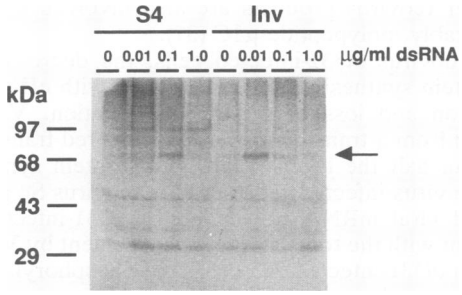


FIG. 4. Increased dsRNA requirement for DAI activation in extracts of cells containing polypeptide σ3. COS-7 cells were transfected with 2.0 μg of pt3S4 (S4) or pt3S4inv (Inv). DAI activity was assayed 48 h later by autophosphorylation in lysates incubated with [γ-$^{32}$P]ATP and dsRNA, immunoprecipitation, and SDS-PAGE. Arrow indicates DAI.

**Phosphorylation of eIF-2α in *dl*331-infected 293 cells.** Inhibition of protein synthesis was essentially complete in 293 cells infected with the adenovirus *dl*331 mutant, and late viral protein synthesis was partially restored by polypeptide σ3 (Fig. 2). It was therefore of interest to test the effect of S4 transfection on eIF-2α phosphorylation in these cells. As shown in Fig. 5 and observed previously (12), eIF-2α was mostly in the phosphorylated state in adenovirus *dl*331-infected 293 cells (lane M). Transfection with pMHVA before infection decreased the proportion of the phosphorylated form of eIF-2α from 85 to 30% (Fig. 5, lane VAI). Transfection with the reovirus S4 gene similarly blocked eIF-2α phosphorylation (Fig. 5, lane S4), although, as already noted, the restoration of viral protein synthesis by polypeptide σ3 in *dl*331-infected 293 cells was less than with VAI RNA.

**Protein synthesis and eIF-2α phosphorylation in reovirus-infected mouse L cells.** Mouse L cells were infected with one of the three reovirus serotypes and labeled with [$^{35}$S]methionine at 21 h postinfection to examine the effects of infection on protein synthesis. As observed in early studies (39), host protein synthesis was decreased by reovirus infection, but the essentially complete shutoff of cellular mRNA translation by type 2 virus reported previously (35) was not apparent (Fig. 6). The level of protein synthesis was similarly and partially diminished in cells infected with reovirus serotype 1, 2, or 3. Consistent with maintenance of cellular protein synthesis, the phosphorylated form of eIF-2α was only slightly increased in reovirus-infected cells (Fig. 7).

## DISCUSSION

Modulation of DAI activity and eIF-2α phosphorylation is a recurrent theme for regulating protein synthesis at the level of initiation. It has been described in animal cells exposed to various conditions of stress including infection with different viruses and recently was shown to mediate *GCN4*-specific translational control in yeast cells starved for amino acids (6). To explore the role of the reovirus S4 gene in the regulation of mammalian cell protein synthesis, we examined the molecular basis for polypeptide σ3 translational enhancement of reporter gene expression in transiently transfected COS cells. The first insights that this viral

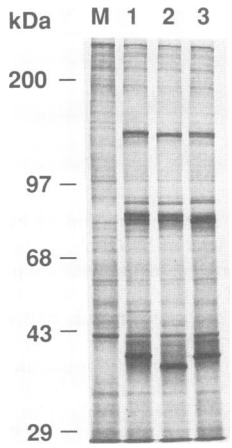


FIG. 6. Analysis of radiolabeled proteins in reovirus-infected mouse L929 cells. Cells were mock infected (lane M) or infected with reovirus serotype 1, 2, or 3 at a multiplicity of infection of 80 PFU per cell (types 1 and 3 were absorbed in 0.1 ml PBS and type 2 was absorbed in 0.5 ml). At 21 h after infection, cells were labeled with [$^{35}$S]methionine, and lysates were analyzed by SDS-PAGE and autoradiography.

dsRNA-binding protein may function to modulate DAI activation came from studies on adenovirus VAI RNA, which also can stimulate the translation of plasmid-derived mRNAs in transfected cells (2, 18, 19). VAI RNA interacts with DAI to block dsRNA activation and prevents eIF-2α phosphorylation and translational inhibition. Cotransfection of a plasmid carrying the reovirus S4 gene substituted for VAI RNA and promoted the expression of reporter DHFR from DAI-activating plasmids (Fig. 1). These results demonstrate that, like VAI RNA, polypeptide σ3 can block DAI activation and preserve initiation of mRNA translation.

The reovirus polypeptide was also shown to replace VAI RNA function in an adenovirus infection. Human 293 cells infected with the VAI-negative adenovirus mutant *dl*331 produced 10-fold-lower amounts of viral proteins than wild-type-infected cells at late times in the replicative cycle. This defect was corrected by transfection before infection with plasmids that express VAI RNA (27). *trans* complementation for viral protein synthesis was also obtained by S4 gene transfection of *dl*331-infected 293 cells (Fig. 2). In contrast to the VAI gene, increasing the S4 DNA did not result in a parallel increase in the synthesis of viral proteins. However, the lower efficiency of enhancement with S4 relative to VAI

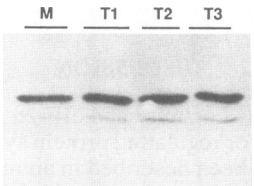


FIG. 7. Phosphorylation state of eIF-2α in reovirus-infected L929 cells. Cells were mock infected (lane M) or infected with reovirus type 1 (lane T1), 2 (lane T2), or 3 (lane T3) at a multiplicity of infection of ~80. Lysates prepared at 21 h postinfection were analyzed by vertical slab isoelectric focusing and Western blotting as described in Materials and Methods. The relative amounts of eIF-2α(P) as measured by densitometry were: lane M, 6%; lane T1, 8%; lane T2, 15%; and lane T3, 10%.

is not surprising since the latter functions at the RNA level, while the S4 effect depends on synthesis of polypeptide σ3 under conditions in which translation is repressed. Although the different nature of the adenovirus and reovirus products, i.e., RNA versus protein, implies that their molecular mechanisms of translational enhancement differ in specific details, it is clear that σ3 can substitute for VAI RNA in a transient expression system that is dependent on DAI inhibition, as well as in adenovirus-infected 293 cells in which viral protein synthesis requires VAI RNA or a VAI RNA-like activity to inhibit DAI.

To measure the effect of σ3 on DAI more directly and to assess its possible significance in reovirus infections, we assayed extracts of infected L cells for DAI activation by autophosphorylation. Maximal levels of activation were dependent on both the time after infection and the amount of dsRNA added to cell extracts, presumably reflecting σ3 content. In addition, the concentration of dsRNA needed for optimal DAI activation was dependent on the viral serotype. Extracts of type 2-infected cells required the lowest level of dsRNA to detect activation of DAI; severalfold more dsRNA was needed to see activation in extracts of cells infected with type 1 virus, and type 3 infections required the highest dsRNA concentration for maximal activation (Fig. 3). In extracts made at earlier times after infection, e.g., 9 h postinfection, DAI was activated by lower levels of dsRNA (24), consistent with the suggestion that the increasing amounts of newly produced viral proteins, notably σ3, influence the levels of DAI activation in reovirus-infected cells. Although it would be of interest to determine directly the levels of endogenously activated DAI in cells infected with the different reovirus serotypes, attempts by $^{32}$P labeling were unsuccessful (24). A relatively low level of σ3 in type 2-infected cells would predict more activated DAI and correlate with the extensive shutoff of host translation reported previously (35). Conversely, the greater interferon resistance of type 1 replication than type 3 replication (16) would imply that there is less activated DAI (and more σ3) in interferon-treated cells infected with type 1 compared with type 3 reovirus.

To target σ3 as a viral product responsible for DAI modulation, we transfected cells with the S4 gene and assayed them for DAI phosphorylation. The presence of the expressed σ3 in extracts of the transfected cells increased the amount of dsRNA necessary for DAI activation by 10-fold compared with cells not containing σ3 (Fig. 4). Although this finding indicates that the presence of σ3 is sufficient to influence DAI activation significantly, it is likely that other reovirus products are also involved in infected cells, notably, polypeptide μ1C (37).

In adenovirus *dl*331-infected cells, the decrease in late viral protein synthesis can be correlated with eIF-2α phosphorylation and loss of translation initiation. VAI RNA produced from a transfected plasmid restored translation to more than half the level of late viral protein synthesis in wild-type virus-infected cells, and the reovirus S4 gene also enhanced viral mRNA translation in *dl*331-infected cells. Consistent with the translational enhancement by VAI RNA and σ3 in *dl*331-infected 293 cells, the phosphorylated form of eIF-2α was decreased nearly threefold by prior transfection with plasmids containing the VAI or S4 gene (Fig. 5). From these results, it seems clear that the presence of σ3 had a protective effect on eIF-2α comparable to that of VAI RNA. This suggests that the molecular basis for the translational stimulation by σ3 in *dl*331-infected 293 cells was at

the level of initiation resulting from a decrease in eIF-2α phosphorylation.

To reconcile the positive translation effects of σ3 with the phenotypic inhibition of host protein synthesis attributed to the S4 gene in reovirus-infected cells (35), it is necessary to consider σ3 as a factor which preserves or restores the translational capacity of infected cells, despite the inhibition of protein synthesis that is part of the antiviral response of the host cell. In effect, as demonstrated in transfected cells, σ3 stimulates the translation of messages that in the absence of the dsRNA-binding polypeptide are repressed by DAI activation. In reovirus-infected cells, the activator of DAI is probably not viral genomic RNA, which apparently exists only in protein complexes as virions or subviral particles (8). The activator in reovirus-infected cells may correspond to duplex regions in the S1 gene transcripts (4). In addition, the σ3 polypeptides derived from each of the three different viral serotypes have translational stimulatory activity (34). Thus, any viral serotype-specific inhibition would depend on the ratio of DAI activator (e.g., S1 mRNA) to translational "stimulator" (polypeptide σ3), and potentially to other products which influence the availability of activator and stimulator. For example, the ability of σ3 to stimulate translation in transfected cells was diminished by coexpression of polypeptide μ1C (37), a viral structural component that forms complexes with σ3 in infected cells (17, 21) and prevents σ3 binding to dsRNA (13). Thus, the ratio of σ3 to μ1C is also likely to be a determinant of DAI activation and translational regulation in reovirus-infected mammalian cells.

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