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Translational Control by Influenza Virus: Suppression of the Kinase That Phosphorylates the Alpha Subunit of Initiation Factor eIF-2 and Selective Translation of Influenza Viral mRNAs

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Selective translation of influenza viral mRNAs occurs after influenza virus superinfection of cells infected with the VAI RNA-negative adenovirus mutant *dl*331 (M. G. Katze, Y.-T. Chen, and R. M. Krug, Cell 37:483–490, 1984). Cell extracts from these doubly infected cells catalyze the initiation of essentially only influenza viral protein synthesis, reproducing the in vivo situation. This selective translation is correlated with a 5- to 10-fold suppression of the *dl*331-induced kinase that phosphorylates the α subunit of eucaryotic initiation factor eIF-2. This strongly suggests that influenza virus encodes a gene product that, analogous to the adenoviral VAI RNA, prevents the shutdown of overall protein synthesis caused by an eIF-2 α kinase turned on by viral infection. Adenoviral mRNA translation was restored to the extract from the doubly infected cells by the addition of the guanine nucleotide exchange factor eIF-2B, which is responsible for the normal recycling of eIF-2 during protein synthesis. This indicates that the residual kinase in the doubly infected cells leads to a limitation in functional (nonsequestered) eIF-2B and hence functional (GTP-containing) eIF-2 and that under these conditions influenza viral mRNAs are selectively translated over adenoviral mRNAs. Addition of double-stranded RNA to the extracts from these cells restored the eIF-2 α kinase to a level approaching that seen in extracts from cells infected with *dl*331 alone and caused the inhibition of influenza viral mRNA translation. This suggests that the putative influenza viral gene product acts against the double-stranded RNA activation of the kinase and indicates that influenza viral mRNA translation is also linked to the level of functional eIF-2. Our results thus indicate that a limitation in functional eIF-2 which causes a nonspecific reduction in the rate of initiation of protein synthesis results in the preferential translation of the better mRNAs (influenza viral mRNAs) at the expense of the poorer mRNAs (adenoviral mRNAs).

Translational control of eucaryotic gene expression, i.e., the selective translation of a specific subset of the available mRNAs, has been studied in several viral and cellular systems (1–3, 6, 29, 37). We have recently demonstrated that influenza virus, a negative-strand RNA virus with a segmented genome, establishes a virus-specific translational control system (15, 16). Although high levels of functional cellular mRNAs are present in the cytoplasm of influenza virus-infected cells, host-cell protein synthesis is effectively shut off, and essentially only viral proteins are synthesized (16).

Selective translation of influenza viral mRNAs also occurs in adenovirus-infected cells which at late times of infection are superinfected with influenza virus (15). In these doubly infected cells, influenza virus-specific proteins are synthesized at essentially the same levels as in cells infected with influenza virus alone, indicating that influenza viral mRNAs can overcome the blocks on nuclear transport and on translation normally exerted by adenovirus on host-cell mRNAs (1, 8). Adenovirus translation also continues at essentially normal levels, indicating that influenza virus does not significantly disturb adenovirus gene expression. Influenza viral mRNA translation in late adenovirus-infected cells is independent of the adenovirus-encoded VAI RNA (26) that is required for adenovirus mRNA translation (36). Thus, after influenza virus superinfection of cells infected with the adenovirus mutant *dl*331, which does not synthesize VAI RNA, influenza viral mRNA translation occurs at high levels, whereas adenovirus mRNA translation remains severely depressed. Because host protein synthesis is also shut off, these doubly infected cells efficiently synthesize essentially only influenza viral proteins.

Recent studies have identified the mechanisms underlying the translational defect in cells infected with *dl*331. Cell extracts from these cells are deficient in functional eIF-2 (27, 33), the initiation factor that forms the ternary complex (eIF-2)-GTP-(Met-tRNA$_i$) that binds to the initiating 40S$_N$ ribosomal subunit before mRNA is bound (12). Addition of stoichiometric amounts of eIF-2 or catalytic amounts of the eIF-2-GTP exchange factor (referred to as eIF-2B) to extracts from *dl*331-infected cells restores translational activity of the endogenous adenovirus mRNAs (27, 33). The inactivation of the eIF-2 in *dl*331-infected cells results from the phosphorylation of its α subunit by a protein kinase activated during infection (33, 35). It has been shown in other systems that such phosphorylation prevents the recycling of eIF-2-GDP to eIF-2-GTP by eIF-2B, the latter being trapped in an inactive complex with eIF-2-GDP (17, 24). Without this recycling of eIF-2 by eIF-2B, protein synthesis initiation is effectively stopped (30). Consequently, at least one role of VAI RNA is to suppress the activity of the eIF-2 α-specific kinase and thus to prevent the nonspecific inhibition of all translation in adenovirus-infected cells.

In the present report, we determined the mechanism by which influenza viral mRNAs are selectively translated

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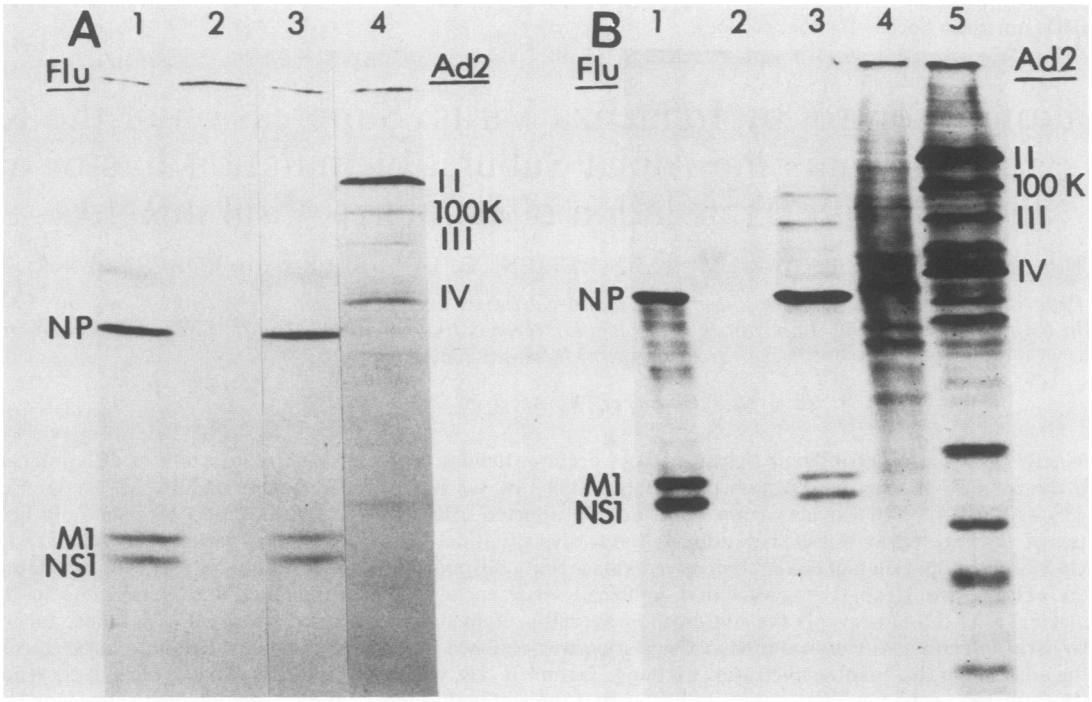

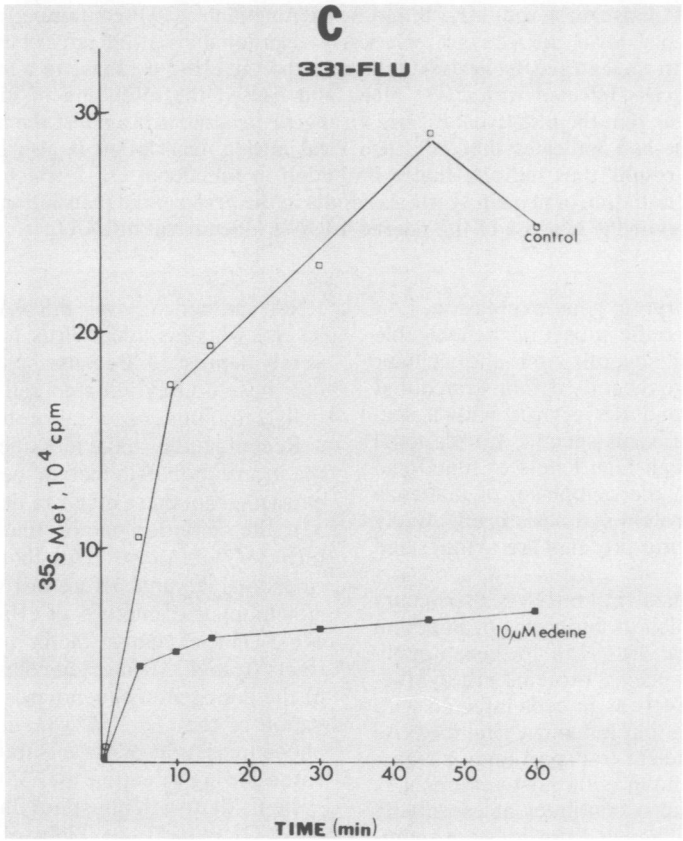

FIG. 1. Influenza virus-specific proteins are selectively translated in cells infected with both influenza virus and *dl*331 and in cell extracts obtained from these cells. (A) Monolayers of 293 cells were infected with adenovirus type 2 (lane 4) or *dl*331 (lane 2) for 20 h or were infected with influenza virus for 4 h (lane 1). Another set of 293 cells was infected with *dl*331 for 16 h and then superinfected with influenza virus for an additional 4 h (lane 3). Each set of cells was subsequently labeled with [^{35}S]methionine for 30 min, and cell-equivalent amounts of the labeled proteins were analyzed on a 14% acrylamide gel. The positions of representative adenovirus- and influenza virus-specific proteins are shown on the right and left, respectively. (B) Proteins synthesized in cell extracts from uninfected and infected 293 cells. Extracts were prepared from mock-infected cells (lane 4), cells infected as described above with influenza virus (lane 1), *dl*331 virus (lane 2), and adenovirus type 2 (lane 5), and cells doubly infected with both *dl*331 and influenza virus (lane 3). In vitro translation reactions as described in the Materials and Methods were carried out at 30°C for 60 min in the presence of [^{35}S]methionine. Labeled proteins were analyzed on a 14% gel. (C) Translational extracts from cells infected with both *dl*331 and influenza virus were incubated in the absence or presence of 10 μM edeine. At the times indicated, samples were removed, and acid-precipitable radioactivity was determined.

despite activation of the eIF-2 α-specific kinase by the adenovirus mutant *dl*331.

MATERIALS AND METHODS

Cells and virus. The adenovirus type 5-transformed human embryonic kidney cell line, 293 (9), was grown in monolayer in Dulbecco modified Eagle medium containing 10% calf serum. Wild-type adenovirus type 2 was purified from infected cells as previously described (25). The adenovirus type 5 deletion mutant *dl*331 (36) was propagated in 293 cells. The titer of adenovirus stocks was determined by plaque assays on 293 cells as described by Lawrence and Ginsberg (19). The WSN strain of influenza A virus was grown in MDBK cells and titrated by plaque assays in MDCK cells as described previously (7).

Virus infection. Monolayer 293 cells were infected with 5 to 10 PFU of adenovirus type 2 or *dl*331 virus per cell for 20 h or with 50 PFU of influenza virus per cell for 4 h. When indicated, *dl*331-infected cells at 16 h of infection were superinfected with influenza virus for an additional 4 h as previously described (15). To analyze protein synthesis in vivo, we labeled cells for 30 min with [^{35}S]methionine (50 μCi/ml) in Dulbecco modified Eagle medium containing 1/10th the normal amount of methionine, and the labeled proteins were subjected to gel electrophoresis as previously described (15).

Cell-free translation. Cell extracts were prepared from 293 cells according to a modification of the protocol of Reichel et al. (27). Cells were washed with ice-cold phosphate-buffered saline and suspended in one pellet volume of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5)–10 mM KCl–1.5 mM magnesium acetate–1 mM dithiothreitol–RNAsin (300 units/ml)–soybean trypsin inhibitor (1 mg/ml). After swelling for 5 to 10 min on ice, the cells were disrupted by homogenization in a type A Dounce homogenizer. The extracts were subjected to two successive centrifugations: 10 min at 4,000 × *g* and 15 min at 13,000 × *g*. The resulting supernatants were utilized in translation assays. Extracts were used the same day they were prepared, as their ability to initiate protein synthesis was diminished during storage in liquid nitrogen. Cell-free translations normally contained approximately 100 μg of protein from the cellular extract, 15 mM HEPES (pH 7.5), 200 mM potassium acetate, 2.25 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 10 mM creatine phosphate, 250 μM spermidine, 50 μg of creatine phosphokinase per ml, 50 μM each L-amino acid (except methionine), and 20 μCi of [^{35}S]methionine. When indicated, the S100 postribosomal supernatant from reticulocyte lysate (23), guanine nucleotide exchange factor, eIF-2B, purified from rabbit reticulocyte lysates as described below, or double-stranded RNA [poly(I)-poly(C)] (dsRNA) was added. The reactions were incubated at 30°C for 60 min, and the translation products were analyzed on 14% polyacrylamide gels.

Protein kinase assays. To remove endogenous ATP, the extracts prepared as described above were extensively dialyzed at 4°C against buffer containing 20 mM Tris hydrochloride (pH 7.5), 50 mM KCl, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, and 5% glycerol. Reactions were carried out as described by Siekierka et al. (35). Reaction mixtures (30 μl) contained the indicated amount of the cellular extract, 20 mM Tris hydrochloride Tris (pH 7.5), 2 mM 2-mercaptoethanol, 4 mM magnesium acetate, and 1 mM [γ-^{32}P]ATP (1,500 cpm/pmol). Highly purified reticulocyte eIF-2 (0.8 μg) prepared as described below was added to reaction mixtures as indicated. When indicated, poly(I)-poly(C) was added at a concentration of 0.15 to 1 μg/ml. After incubation for 6 min at 30°C, the reactions were terminated by the addition of an equal volume of twofold-concentrated gel buffer and were immediately boiled before being subjected to polyacrylamide gel electrophoresis.

Phosphatase assays. Purified rabbit reticulocyte eIF-2 was phosphorylated with partially purified eIF-2 α and β subunit kinases from reticulocyte lysate and [γ-^{32}P]ATP (31) and separated from labeled ATP by gel filtration on Sephadex G-50. This [^{32}P]eIF-2α,β was incubated with 13 μg of dialyzed cell extract in a final volume of 50 μl under the kinase assay conditions described above except that the ATP was unlabeled. After the indicated times at 30°C, samples were removed and added to an equal volume of twofold-concentrated gel buffer at 100°C. Labeled proteins were subjected to polyacrylamide gel electrophoresis as described above.

Eucaryotic initiation factors. The initiation factors eIF-2 and eIF-2B were purified by a modification of the procedure of Konieczny and Safer (17) to be published elsewhere. Briefly, rabbit reticulocyte lysate was bound to phosphocellulose and batch eluted with 0.7 M KCl, and this eluate was applied to DEAE-cellulose. After elution with a linear 0.1 to 0.5 M KCl gradient, the overlapping free eIF-2 and eIF-2-eIF-2B complex pools were then resolved into highly purified eIF-2 and eIF-2B by Mono Q FPLC chromatography (Pharmacia, Inc., Piscataway, N.J.).

RESULTS

Protein synthetic activity of extracts. To investigate the mechanisms by which influenza viral mRNAs are selectively translated in late adenovirus-infected cells in the absence of VAI RNA, it was necessary to prepare cell-free translational extracts that faithfully reproduce in vivo events. Figure 1A shows the in vivo pattern of protein synthesis in 293 cells. Little or no protein was made in *dl*331-infected cells (lane 2) relative to that made in cells infected with wild-type adenovirus (lane 4). In contrast, efficient synthesis of essentially only influenza viral, and not adenoviral, proteins occurred in cells infected with both *dl*331 and influenza virus (lane 3), as shown previously (15). The amount of influenza virus-specific protein synthesis in the doubly infected cells was slightly reduced relative to that in the cells infected with influenza virus alone. This reduction mainly manifested itself in the levels of M1 and NS1 synthesis. In Fig. 1A, M1 and NS1 synthesis was reduced by 15 to 20%, as measured by densitometer tracing of the autoradiogram. However, in other experiments, this reduction has been as much as 40%.

Figure 1B shows the proteins made in vitro by cytoplasmic extracts from 293 cells. The extracts from *dl*331-infected 293 cells synthesized little or no protein (lane 2) compared with extracts from uninfected cells (lane 4) and from cells infected with wild-type adenovirus type 2 (lane 5). Thus, the translational defect of *dl*331-infected cells is preserved in vitro, as shown by others (27, 32). In contrast, the extracts from cells infected with both *dl*331 and influenza virus efficiently synthesized predominantly influenza viral proteins in vitro (lane 3), again reproducing the in vivo results. As was the case in vivo, the protein-synthesizing activity of the extract from the doubly infected cells was reduced by about 25 to 50% relative to that of the extract from cells infected with influenza virus alone (lane 1), as determined both by densitometer tracing of the autoradiogram and by measuring the total incorporation of [^{35}S]methionine into acid-

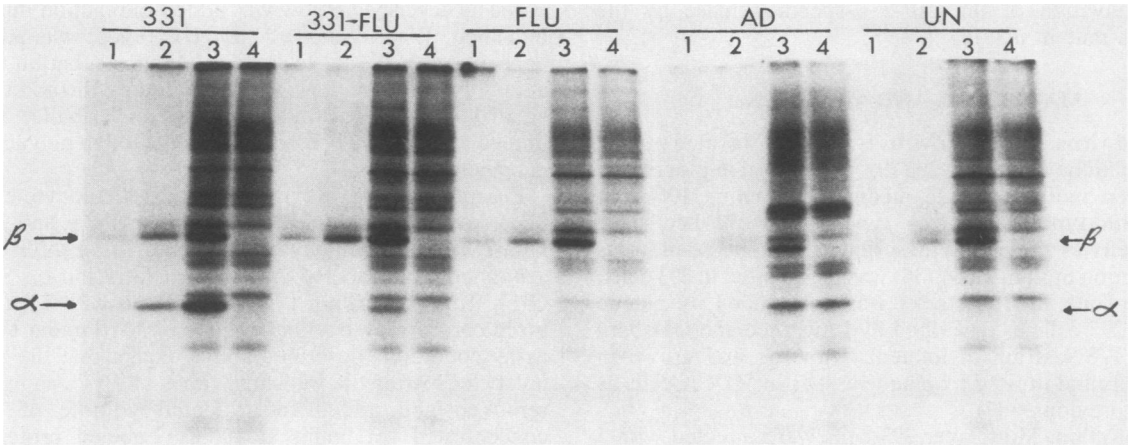

FIG. 2. Influenza virus superinfection suppresses the high eIF-2 α protein kinase level induced after infection with *dl*331. Cell extracts were prepared as described in the Materials and Methods from cells infected with *dl*331 alone (331), with both *dl*331 and influenza virus (331-FLU), with influenza virus alone (FLU), with adenovirus type 2 (AD), and from uninfected cells (UN). Reaction mixtures carried out as described in the Materials and Methods contained 0.8 μg of purified reticulocyte eIF-2 and the following amounts of extracts: lane 1, 0.5 μg; lane 2, 2.0 μg; lane 3, 8.0 μg. Lane 4 is a reaction containing 8.0 μg of the indicated extract incubated in the absence of added eIF-2. Reaction mixtures were incubated for 6 min at 30°C, and the labeled proteins were resolved by electrophoresis on a 14% polyacrylamide gel. Arrows indicate the positions of the α and β subunits of eIF-2 which were determined by staining the gel with Coomassie blue.

precipitable products. This reduction is most evident in the relative levels of M1 and NS1 protein synthesis catalyzed by the two extracts. The reason for this reduced protein synthetic activity will be shown later.

To determine whether the protein synthesis catalyzed by the extract from the doubly infected cell was due to initiation as well as elongation of chains, we determined the sensitivity of this synthesis to 10 μM edeine (Fig. 1C). Almost all the synthesis after 5 min of incubation was edeine sensitive, and the overall edeine sensitivity was about 70 to 80%. This indicates that high levels of initiation are occurring in vitro. Similar edeine sensitivity was observed with the extract from cells infected with influenza virus alone or with wild-type adenovirus and with the extract from uninfected cells. These data indicate that the effects of influenza virus infection on the translational machinery of *dl*331-infected cells can be studied in vitro.

Influenza virus superinfection suppresses the high levels of eIF-2 α kinase induced after infection with *dl*331 virus. It has recently been shown that *dl*331-infected cells, but not wild-type adenovirus-infected cells, contain high levels of a protein kinase that phosphorylates the α subunit of eIF-2 (33, 35). This almost certainly causes the dramatic inhibition of the initiation of protein synthesis in *dl*311-infected cells. Since influenza viral mRNAs are efficiently translated in these cells, we determined whether influenza virus superinfection of *dl*331-infected 293 cells reduced the level of this kinase activity. We confirmed that extracts from *dl*331-infected cells contain a high level of kinase that phosphorylates the α subunit of eIF-2 (Fig. 2). Detection of this kinase was dependent on the addition of exogenous eIF-2, as phosphorylation of the α subunit of the endogenous eIF-2 was not detectable (lane 4). After superinfection with influenza virus, the kinase specific for the α subunit of eIF-2 was reduced 5- to 10-fold relative to that found in extracts from cells infected with *dl*331 alone. Influenza virus caused a similar suppression of the *dl*331-induced eIF-2 α kinase in monolayer HeLa cells (data not shown). In contrast to the eIF-2 α kinase, the kinase that phosphorylates the β subunit of eIF-2 was not significantly affected by influenza virus superinfection of *dl*331-infected cells. In other systems also, this β kinase is apparently not regulated (12). Little kinase specific for the α subunit of eIF-2 was observed in extracts from cells infected with wild-type adenovirus (Fig. 2), as shown previously by others (33, 35), because VAI RNA totally suppresses this kinase. In addition, little of this kinase was observed in extracts from cells infected with influenza virus alone and in extracts from uninfected cells.

One possibility was that the apparent suppression of the *dl*331-induced eIF-2 α kinase by influenza virus was attributable to an influenza virus-induced increase in the activity of an eIF-2 α-specific phosphatase. To assess this possibility, we measured this phosphatase activity in extracts from 293 cells infected either with *dl*331 alone or with both *dl*331 and influenza virus (Fig. 3). Exogenous [^{32}P]eIF-2α,β was added to these extracts under the same conditions as employed for the kinase assays. At the level of extract used in this experiment, which was comparable to the highest level used in the kinase assays, only a very small amount of dephosphorylation of the α subunit was observed with either extract after 6 min of incubation at 30°C (the same incubation period as the kinase assays). Densitometer tracing showed that the reduction in label in the α subunit was only 20 to 25% with either extract. In contrast, reticulocyte lysate contained a high level of a phosphatase specific for eIF-2 α, as previously shown (31). With longer incubations (30 min) with the infected-cell extracts, the reduction in the α-subunit label reached 50 to 60%, but the two extracts behaved similarly (data not shown). Thus, the reduction in the ability of the extract from cells infected with both *dl*331 and influenza virus to phosphorylate the α subunit of eIF-2 is due to suppression of the kinase rather than to activation of a phosphatase.

These results indicate that influenza virus encodes a gene product that, analogous to the adenovirus VAI RNA, prevents the inactivation of eIF-2 by a protein kinase activated during virus infection.

eIF-2B restores adenoviral mRNA translation. The suppression by influenza virus of the high kinase activity specific for the α subunit of eIF-2 can explain why influenza

viral protein synthesis is efficiently initiated in cells infected with both *dl*331 and influenza virus. The question then is why the doubly infected cells, both in vivo and in vitro, support essentially only influenza viral and not adenoviral mRNA translation. One possibility is that the residual kinase level in the doubly infected cells results in sufficient sequestering of eIF-2B to make free eIF-2B limiting and hence functional eIF-2 (i.e., eIF-2 containing GTP) limiting. Influenza viral mRNAs might then outcompete the adenovirus mRNAs for preinitiation complexes containing functional eIF-2.

Figure 4 shows the experiment to test this possibility. Others have shown that when eIF-2 or eIF-2B is added to an extract from *dl*331-infected cells, a substantial amount of adenovirus translation is restored (27, 33). For our experiments, we used purified eIF-2B free of any detectable eIF-2, so that any stimulation of protein synthesis that occurred after the addition of this initiation factor could be attributed only to a deficiency in the level of the endogenous functional (i.e., nonsequestered) eIF-2B in the extracts. The purity of our eIF-2B preparation was established by analysis on gels (Fig. 4B): the five subunits of eIF-2B were essentially the only proteins detected by silver staining. Addition of this pure eIF-2B to extracts from *dl*331-infected cells (Fig. 4A) stimulated the synthesis of the high-molecular-weight adenovirus proteins, e.g., II and 100K, at least fivefold (Fig. 4C). The stimulation of the synthesis of the lower-molecular-weight adenovirus proteins was much greater, as these proteins could only be detected after the addition of eIF-2B (Fig. 4A). This is the case even with much longer exposures of the autoradiogram.

When the pure eIF-2B was added to the extracts from cells infected with both *dl*331 and influenza virus, there was only a small effect on influenza virus translation: M1 and NS1 synthesis was increased slightly, to a level approaching that seen with the extracts from cells infected with influenza virus alone. With the latter extracts, influenza viral mRNA translation was not detectably affected by eIF-2B addition. With the extracts from the doubly infected cells, the most striking effect of eIF-2B addition was on adenovirus translation, which was stimulated to the same extent as with the extracts from cells infected with *dl*331 alone. Again, the synthesis of the high-molecular-weight adenovirus proteins was stimulated at least fivefold (Fig. 4C), and a much greater stimulation of the synthesis of the lower-molecular-weight adenovirus proteins was observed (Fig. 4A). As verified by the densitometer tracing of the high-molecular-weight proteins (Fig. 4C), the synthesis of adenovirus proteins II and 100K was stimulated to the same extent in the two extracts. In addition, the S100 fraction from reticulocyte extracts, which contains large amounts of eIF-2B (27), stimulated the synthesis of II and 100K to a greater extent than the pure eIF-2B, but most importantly stimulated this synthesis to the same extent in the two extracts.

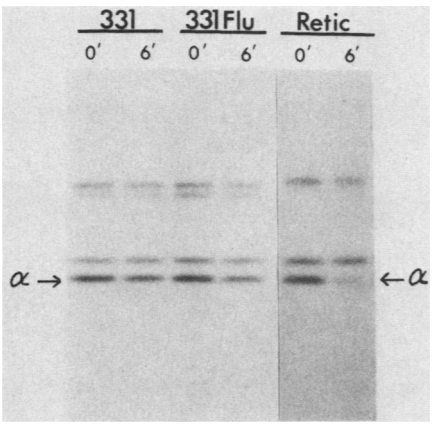

FIG. 3. eIF-2 α-specific phosphatase activity is not increased after superinfection with influenza virus. [^{32}P]eIF-2α,β was incubated with cell extracts from 293 cells infected with *dl*331 alone (331) or with both *dl*331 and influenza virus (331Flu). A reaction containing reticulocyte lysate was included as phosphatase-positive control (31). Samples were removed at 0 and 6 min and processed as described in the Materials and Methods. Labeled proteins were analyzed on a 14% polyacrylamide gel. The arrow indicates the position of the α subunit of eIF-2. The other phosphorylated bands seen in the gel are the β subunit of eIF-2 (uppermost band) and two other phosphorylated proteins that originate from the partially purified reticulocyte kinase used to phosphorylate the purified eIF-2 substrate.

These results indicate that functional adenoviral mRNAs are present in the extracts of the doubly infected cells but that they are not translated because of a deficiency in the level of functional (nonsequestered) eIF-2B and hence functional eIF-2. In contrast, the influenza viral mRNAs present in the extracts are selectively translated in the presence of limiting functional eIF-2, although the synthesis of the M1 and NS1 proteins is slightly compromised.

dsRNA activates the kinase and suppresses influenza viral mRNA translation. To verify that the translational selectivity operates via the level of functional eIF-2, it was essential to establish that influenza virus translation was tightly linked to the level of the kinase and hence of functional eIF-2. In other words, if the kinase level in the extracts from the doubly infected cells was increased, then influenza viral mRNA translation should be effectively inhibited.

This issue could be addressed because of the effect of low amounts of dsRNA [poly(I)-poly(C)] on the kinase level. The rationale for adding dsRNA was based on the presumption that the *dl*331-induced kinase is the same as the interferon-induced kinase that is activated by dsRNA (20). Addition of dsRNA to extracts from cells infected with *dl*331 alone resulted in only about a 50% stimulation of the kinase specific for eIF-2 α (Fig. 5), as determined by densitometer tracing; in other experiments this stimulation was as much as twofold. This suggests that this kinase was already largely activated by dsRNA produced during adenovirus infection. In contrast, addition of dsRNA to the extracts from cells infected with both *dl*331 and influenza virus caused a 5- to 10-fold activation of the eIF-2 α kinase. This activation occurred with as little as 0.15 μg of dsRNA per ml. The resulting kinase level approached that seen in the extracts from cells infected with *dl*331 alone. These results suggest that the putative influenza virus gene product acts against the dsRNA activation of the kinase. It should be noted that dsRNA did not cause a significant increase in the level of eIF-2 α kinase activity in extracts from uninfected cells, indicating that the level of the endogenous kinase in 293 cells was very low relative to that seen after *dl*331 infection. Neither did dsRNA significantly affect the kinase level in 293 cells after influenza virus infection nor after wild-type adenovirus infection.

Because the addition of dsRNA activated the kinase in extracts from the doubly infected cells, the amount of functional eIF-2 should be substantially reduced, and as a consequence influenza viral mRNA translation should be inhibited. Extracts from cells infected with influenza virus alone should serve as a good control; since dsRNA did not

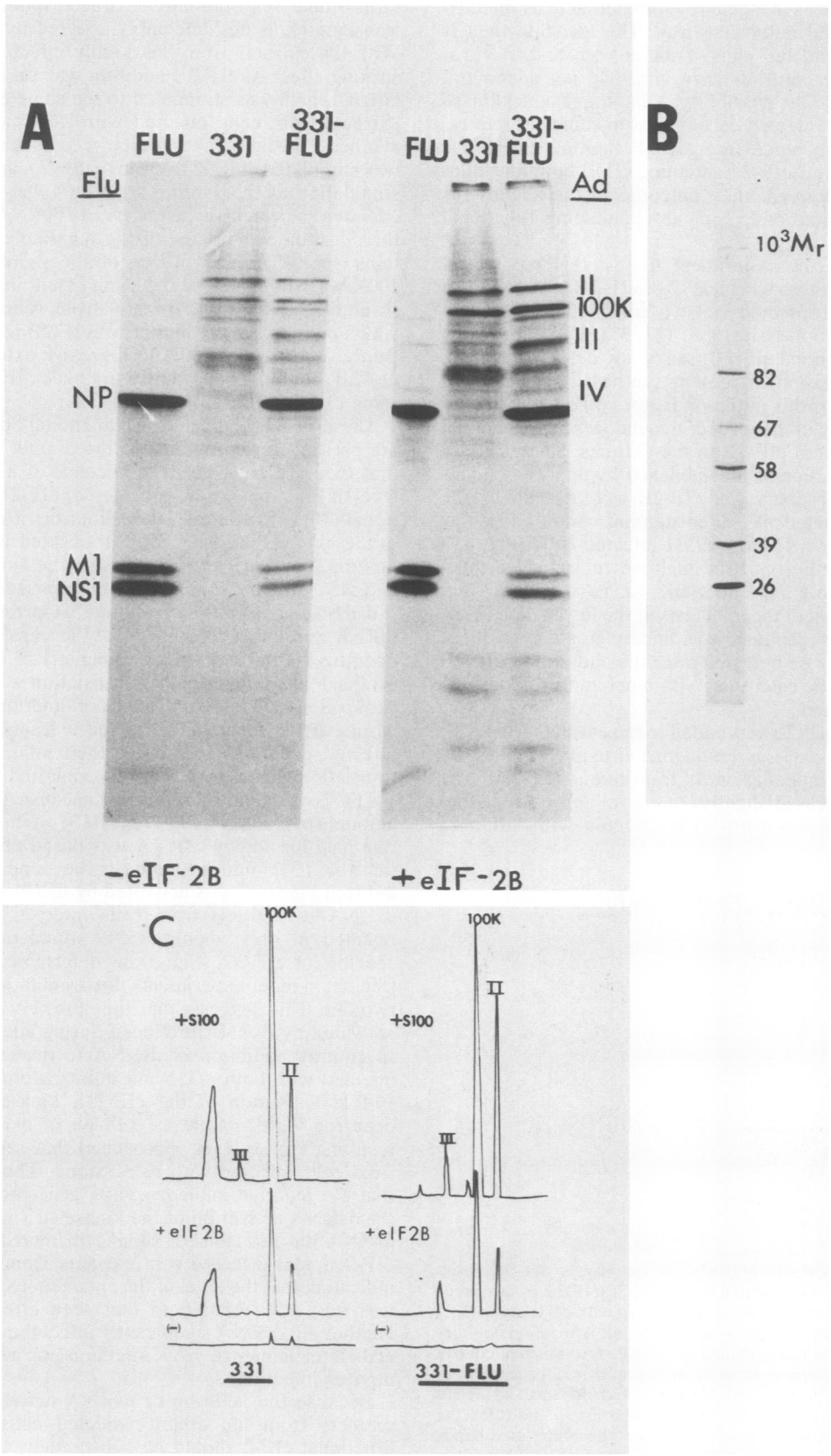

FIG. 4. eIF-2B restores adenovirus translational activity to *dl*331 and *dl*331-influenza virus extracts. (A) Cell extracts were prepared from cells infected with influenza virus alone (FLU), with *dl*331 alone (331), or with both *dl*331 and influenza virus (331-FLU). In vitro translation reactions were carried out at 30°C for 75 min, either unsupplemented (−eIF-2B) or supplemented (+eIF-2B) with purified guanine nucleotide

significantly increase the eIF-2 α kinase activity, influenza viral mRNA translation catalyzed by these extracts should not be affected by dsRNA addition. The predicted results were obtained (Fig. 6). With the extracts from the cells infected with both *dl*331 and influenza virus, the inhibition of NP, NS1, and M1 synthesis caused by the addition of dsRNA was about 50% and thus approached the maximum inhibition of initiation possible in these extracts, namely, 70% as determined by edeine sensitivity. Kinase assays confirmed that in these translation reactions dsRNA caused a 5- to 10-fold activation of the eIF-2 α kinase. In contrast, with the extracts from cells infected with influenza virus alone, dsRNA had almost no effect (at most a 5 to 10% inhibition) on the synthesis of NP, NS1, and M1, again as compared with a 70% inhibition seen with edeine. We can conclude that the initiation of influenza viral mRNA translation is linked to the level of functional eIF-2, since this translation is effectively inhibited when the eIF-2 α kinase level of the extracts from the doubly infected cells is increased.

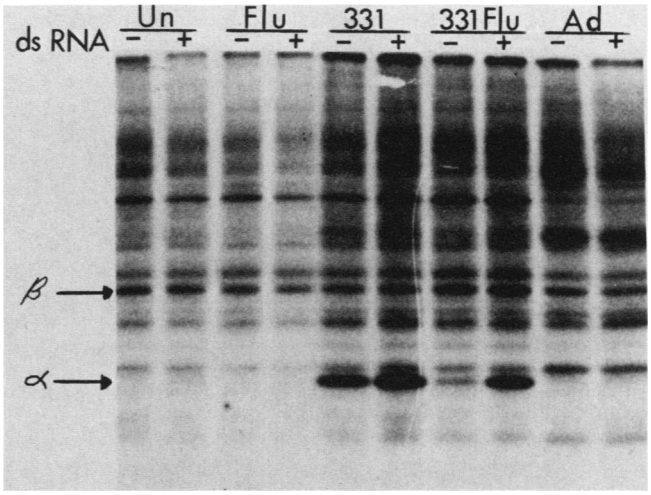

FIG. 5. dsRNA stimulates the activity of the eIF-2 α kinase in *dl*331-influenza virus extracts. Cell extracts were prepared from infected and uninfected cells, and the kinase reaction containing 8.0 μg of the indicated extract was performed as described in the legend to Fig. 2. Purified reticulocyte eIF-2 (0.8 μg) was added to each reaction. Where indicated, poly(I)-poly(C) was added at a concentration of 1 μg/ml. Arrows indicate the positions of the α and β subunits of eIF-2. Designations are as in Fig. 2.

DISCUSSION

When cells infected with the VAI RNA-negative adenovirus type 2 mutant *dl*331 are superinfected with influenza virus, essentially only influenza virus proteins are synthesized (15). We showed that this selective translation most likely results from a partial suppression by influenza virus of the eIF-2 α kinase activity expressed after *dl*331 infection. Promotion of eIF-2 activity by the addition of the factor (eIF-2B) responsible for its normal recycling during protein synthesis initiation was able to restore adenovirus type 2 mRNA translation, and conversely, reduction of eIF-2 activity by the reactivation of the eIF-2 α kinase with dsRNA caused the inhibition of influenza viral mRNA translation. This strongly suggests that the translational selectivity observed in these doubly infected cells in vitro and in vivo is a titratable function of the amount of active eIF-2 available for translational initiation. Consequently, the partial suppression of eIF-2 α kinase activity enables influenza viral proteins to be synthesized. In contrast, with adenovirus mRNA translation, the partial suppression of the kinase is offset by the competition for limiting functional eIF-2 by influenza viral mRNAs, with the net result being that adenovirus mRNA translation remains as inhibited as in cells infected with *dl*331 alone. The questions raised, therefore, are (i) what is the identity and mechanism of action of the influenza viral gene product responsible for suppression of the *dl*331-induced eIF-2 α kinase; (ii) what is the normal function of this influenza viral gene product; (iii) why are influenza viral mRNAs selectively translated over adenoviral mRNAs when functional eIF-2 is limiting; and (iv) why do host mRNAs remain poorly translated?

Our results indicate that the influenza viral gene product acts against the dsRNA activation of the eIF-2 α kinase, since addition of dsRNA to extracts from doubly infected cells restores the kinase to a level approaching that observed in extracts from cells infected with *dl*331 alone. The kinase in the latter extracts is only slightly stimulated by the addition of dsRNA (33). This result has been interpreted as indicating that the kinase in *dl*331-infected cells is dsRNA dependent but that it is almost completely activated by endogenous dsRNA produced during adenovirus infection. Our results prove that this kinase is dsRNA dependent and suggest that the influenza viral gene product reverses the dsRNA activation that occurred during the period of adenovirus infection (16 h) before influenza virus superinfection or prevents further activation by dsRNA or both. This is consistent with the existence of an equilibrium between the inactive and active form of the kinase, with dsRNA causing activation and the influenza viral gene product causing deactivation.

What is the normal function of the influenza virus gene product that suppresses the eIF-2 α-specific kinase? One possibility is that this kinase is normally activated by dsRNAs produced during influenza virus infection and that the virus codes for a product that suppresses this kinase as a mechanism for stopping the phosphorylation of the α subunit of eIF-2 and the consequent general inhibition of protein synthesis. This explains the absence of detectable eIF-2 α kinase activity in extracts from cells infected with influenza virus alone. Indeed, this could be a common mechanism employed by several viruses, e.g., adenovirus, influenza virus, and vaccinia virus. Vaccinia virus apparently codes for a factor that inhibits the interferon-induced eIF-2 kinase (28, 38); this factor could also act against the endogenous kinase present in the absence of interferon treatment. In contrast to these viruses, vesicular stomatitis virus probably

exchange factor eIF-2B (0.15 μg). The polypeptides were analyzed on a 14% polyacrylamide gel. Positions of representative adenovirus- and influenza virus-specific proteins are shown on the right and left, respectively. (B) The purified eIF-2B was electrophoresed on a 14% acrylamide gel containing sodium dodecyl sulfate, and the gel was then stained with silver to identify the protein bands. (C) Densitometer tracing of the high-molecular-weight adenovirus proteins synthesized in extracts prepared from cells infected with *dl*331 alone (331) and with both *dl*331 and influenza virus (331-FLU). (−) indicates no addition to the extracts; +eIF-2B indicates extracts supplemented with the purified initiation factor; +S100 indicates extracts supplemented with the postribosomal supernatant from micrococcal nuclease-treated reticulocyte lysate.

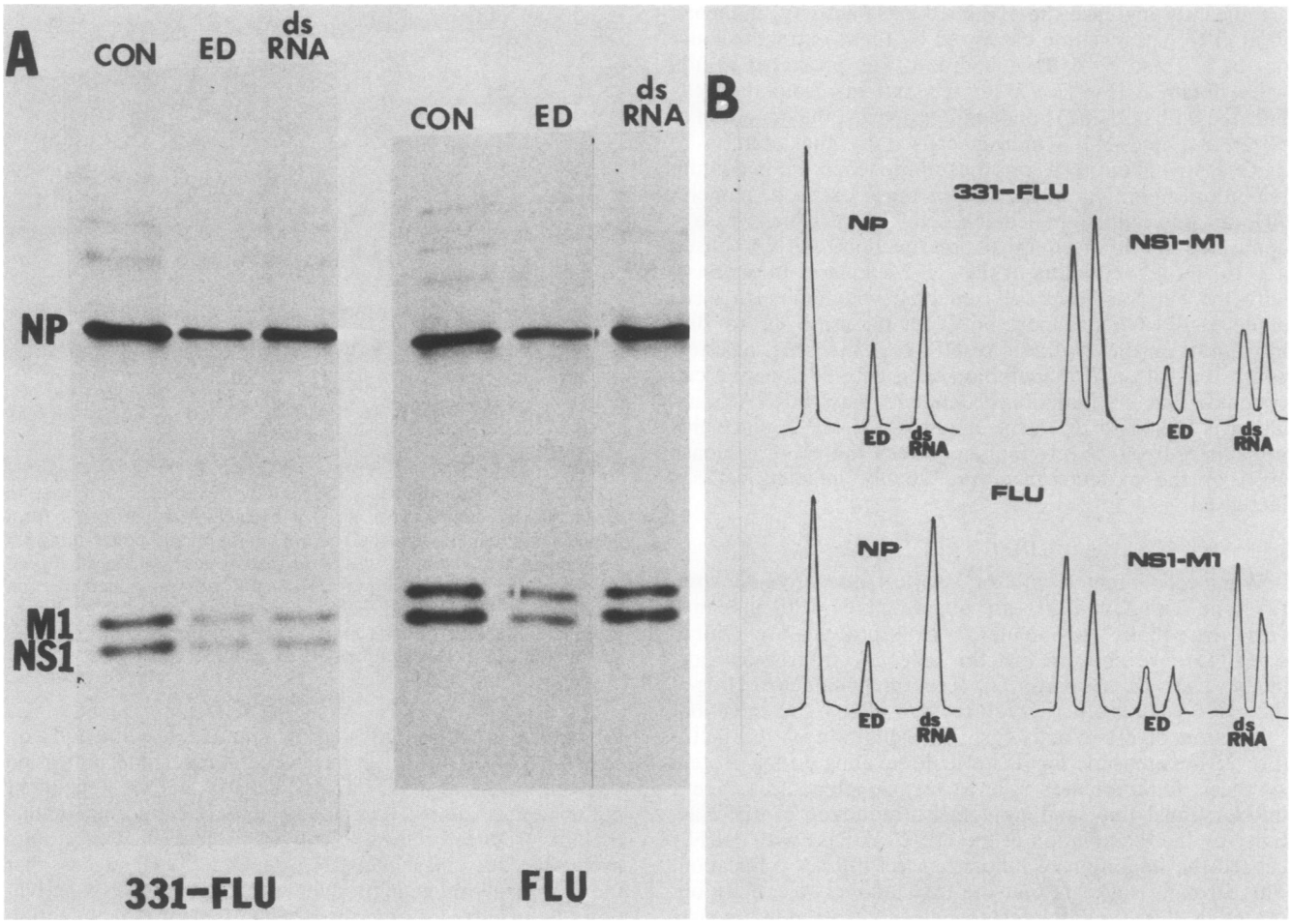

FIG. 6. dsRNA inhibits the translation of influenza viral mRNAs in *dl*331-influenza virus extracts. (A) Cell extracts were prepared from cells infected with influenza virus alone (FLU) or with both *dl*331 and influenza virus (331-FLU). In vitro translation reactions were carried out at 30°C for 60 min. Extracts were run either unsupplemented as a control (CON); supplemented with edeine at a concentration of 10 μM (ED); or supplemented with poly(I)-poly(C) at a concentration of 0.4 μg/ml (dsRNA). Labeled proteins were analyzed on a 14% polyacrylamide gel. (B) Densitometer tracing of the NP and NS1-M1 proteins synthesized in extracts from *dl*331-influenza virus-infected cells (331-FLU) or from cells infected with influenza virus alone (FLU). The tracings were made from a lighter exposure of the autoradiogram shown in panel A.

does not code for a product that suppresses this kinase, as the levels of eIF-2 α phosphorylation are high and overall protein synthesis is inhibited after vesicular stomatitis virus infection (4). It should be pointed out that there may be fundamental differences in the levels of the kinase after infection with these different viruses. For example, adenovirus may actually induce the synthesis of more kinase as well as provide the dsRNA that activates the kinase, whereas other viruses (including influenza virus) may only activate the endogenous kinase by producing dsRNA.

In addition, a gene product that suppresses the kinase would afford protection against the action of interferon, as the kinase induced in *dl*331-infected cells is probably the same as the dsRNA-dependent kinase induced after interferon treatment (20). With influenza virus, efficient inhibition of viral replication by interferon α/β is dependent on the presence of the cellular Mx gene (10). This interferon-induced gene encodes a 75,000-molecular-weight protein that accumulates in the nucleus (5, 11). In cells containing the Mx gene, interferon treatment results in a dramatic inhibition of viral mRNA synthesis in the nucleus (18). In contrast, in cells lacking the Mx gene, viral mRNA synthesis is normal while viral protein synthesis is delayed relative to that occurring in the absence of interferon (18). This delay could reflect the time required for the putative viral gene product to suppress the interferon-induced, dsRNA-dependent eIF-2 kinase. We are currently testing this possibility.

The eIF-2 kinase activity induced after *dl*331 infection is suppressed, but not eliminated, by influenza virus superinfection, and our results provide strong evidence that the residual kinase is largely responsible for the selective translation of influenza viral over adenoviral mRNAs in these doubly infected cells. Addition of eIF-2B to extracts from these cells restores adenovirus translation, and the extent of this restoration is identical to that found after eIF-2B addition to extracts from cells infected with *dl*331 alone. Because eIF-2B addition eliminates any limitation in functional eIF-2 (i.e., eIF-2 containing GTP), it can be concluded that influenza viral mRNAs are selectively translated over adenoviral mRNAs in the presence of limiting functional eIF-2.

There are several possible explanations for this selectiv-

ity. First, it might be due to a large excess of influenza viral over adenoviral mRNAs in the doubly infected cells. This is almost certainly not the case. We had previously found similar levels of adenoviral and influenza viral mRNAs in cells infected with both influenza virus and wild-type adenovirus (15), and we have recently found that similar levels of these two sets of mRNAs are also found in cells infected with both influenza virus and *dl*331 (data not shown). A second possibility is that influenza viral mRNAs might have a higher affinity for eIF-2 than adenoviral mRNAs. However, this is not likely because it is generally felt that eIF-2, particularly as it acts in translation before mRNA binding, does not exhibit mRNA preferences (12), although one group of investigators has presented evidence that certain mRNAs possess a high-affinity binding site for eIF-2 (13, 14). The third and most likely possibility is that influenza viral mRNAs are simply better initiators of translation than adenoviral mRNAs and as a consequence preferentially interact with the limiting amount of eIF-2-containing initiation complexes on the ribosomes. The model for protein synthesis described by Lodish (21, 22) predicts that any nonspecific "reduction in the rate of polypeptide chain initiation steps at or before binding of mRNA will result in preferential inhibition of translation of mRNAs with lower rate constants for polypeptide chain initiation (the poorer mRNAs)." Our results provide strong evidence that a limitation in functional eIF-2 constitutes such a nonspecific reduction in the rate of initiation that results in the preferential translation of the better mRNAs (influenza viral mRNAs) at the expense of the poorer mRNAs (adenoviral mRNAs). A limitation, rather than elimination, in functional eIF-2 may be relatively unique to the cells infected with both *dl*331 and influenza virus. In these cells the residual phosphorylation of eIF-2 apparently causes the sequestration of some, but not all, of the available eIF-2B, leading to only a 25 to 50% inhibition of overall protein synthesis. Clearly, it will be important to determine what fraction of the endogenous eIF-2 in the doubly infected cells contains a phosphorylated α subunit and to ascertain the relative size of the nonsequestered eIF-2B pool. In contrast, in other systems in which phosphorylation of the α subunit of eIF-2 has been described, almost all of the available eIF-2B is probably sequestered, and the inhibition of overall protein synthesis is almost complete (17, 24, 34).

An important question is whether the results obtained with the cells infected with both *dl*331 and influenza virus shed light on the mechanism of selective shutoff of host-cell protein synthesis in cells infected with influenza virus alone. It appears that this shutoff is not related to levels of functional eIF-2 since: (i) little or no eIF-2 α kinase is induced in cells infected with influenza virus alone (Fig. 2 and 5); and (ii) the addition of purified eIF-2B to extracts from cells infected with influenza virus alone does not restore detectable amounts of host-cell translation (Fig. 4). It is a distinct possibility, therefore, that alteration of initiation factors other than eIF-2 may be involved in the normal mechanism(s) of shutoff of host-cell translation during infection. In addition, host-cell translation could be blocked at the level of elongation of polypeptide chains. In at least one other system, i.e., after induction of the stress response in cells by heat shock or amino acid analogs, the rate of both initiation and elongation of polypeptide chains encoded by the untranslated (i.e., nonstress) mRNAs is greatly reduced (2, 37). We are currently examining the polyribosomal distribution of host-cell mRNAs after influenza virus infection to determine whether the block in host-cell translation is at the level of initiation or elongation or both. Influenza virus may act at several levels to ensure both the efficient and the selective translation of its mRNAs.

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