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Translational Control Mediated by Eucaryotic Initiation Factor-2 Is Restricted to Specific mRNAs in Transfected Cells

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The translational efficiency of mRNA molecules transcribed from plasmid DNA transfected into COS-1 monkey cells can be increased 10- to 20-fold by the coexpression of the adenovirus virus-associated RNAs I and II. Experiments described here demonstrate a similar increase in translational efficiency by the addition of 2-aminopurine, an inhibitor of double-stranded RNA-activated protein kinase, to the culture medium. Both virus-associated RNA and 2-aminopurine presumably exert their effect by alteration of the functional level of eucaryotic initiation factor-2. The translational stimulation mediated by both means is shown to be restricted to the plasmid-derived mRNAs because there is no qualitative or quantitative alteration in host protein synthesis. The results are consistent with models invoking a localized activation of double-stranded RNA-activated kinase leading to a translational block.

To initiate polypeptide chain synthesis, eucaryotic initiation factor-2 (eIF-2) forms a ternary complex with GTP and initiator Met-tRNA (for reviews, see references 14 and 15). The ternary complex binds a 40S ribosomal subunit, forming a 40S initiation complex. Subsequently, an mRNA molecule and the 60S ribosomal subunit are added to form the 80S initiation complex, with the concomitant hydrolysis of GTP to GDP. To reinitiate, GDP bound to eIF-2 must be replaced by GTP, a reaction catalyzed by the guanine nucleotide exchange factor. The phosphorylation of the alpha subunit of eIF-2 results in stabilizing the guanine exchange factor–eIF-2–GDP complex, thereby inhibiting recycling. This depletes the amount of functional eIF-2 competent for translation initiation.

Two protein kinases have been shown to regulate initiation by phosphorylation of the alpha subunit of eIF-2 (15). The hemin-controlled inhibitor of protein synthesis has been studied in reticulocytes and is activated by various stimuli including hemin deprivation and heat treatment (6, 19). The double-stranded RNA (dsRNA)-activated inhibitor (DAI) is induced by interferon, and its activity is dependent on dsRNA (12, 18, 20, 31). Translational control mediated by DAI protein kinase has also been studied in adenovirus-infected cells, in which it has been found that two RNA polymerase III transcripts from the adenovirus genome are expressed at high levels and are required for efficient translation of adenovirus mRNAs late after infection (27). The virus-associated (VA) RNA I is involved in the control of the level of active eIF-2 (17, 21–23). VA RNA I can inhibit the activation of DAI kinase by dsRNA in vitro (11, 16). It has been proposed that dsRNA which may result from asymmetric transcription of the replicating adenoviral genome in infected cells can activate DAI kinase to phosphorylate the alpha subunit of eIF-2, resulting in inhibition of translation initiation. VA RNA may prevent the translational block by inhibiting activation of DAI kinase. In addition to the role of VA RNA in adenovirus-infected cells, VA RNA can potentiate the translation of mRNA in transient transfection experiments (7, 25, 26). Although the role of VA RNA is not well understood in transient DNA transfection, it is probable that VA RNA is responsible for promoting translation by preventing dsRNA activation of the DAI kinase.

If VA RNA acts by inhibition of DAI kinase in transient transfection experiments, then 2-aminopurine, a relatively specific inhibitor of DAI kinase in vitro (3), should exhibit a similar effect. The effect of VA RNA and 2-aminopurine in potentiation of mRNA translation in transfected COS cells is shown in Fig. 1. A plasmid was constructed to express murine adenosine deaminase (ADA) from the adenovirus major late promoter, from an mRNA that contains the majority of the adenovirus tripartite leader sequence which is present on adenovirus late mRNAs. The plasmid was constructed with (p9ADA5-29) and without (p9A) the presence of the adenovirus VA RNA genes I and II (Fig. 1a). Transfection of COS-1 cells yielded approximately 10-fold greater levels of ADA synthesis when the VA genes were present (Fig. 1b, compare lanes 3 and 5). The bands migrating at 43 and 48 kilodaltons were shown to be ADA by immunoprecipitation with ADA-specific antisera (data not shown). The addition of 10 mM 2-aminopurine to cells transfected with the VA deficient expression plasmid resulted in a 12-fold increase in ADA translation (Fig. 1b, compare lanes 5 and 6). Lower concentrations of 2-aminopurine had a lesser effect (Fig. 1b, lane 7). 2-Aminopurine also increased translation of ADA in cells transfected with the VA RNA genes containing plasmid p9ADA5-29 (Fig. 1b, compare lanes 3 and 4). ADA synthesis in the 2-aminopurine-treated cells represented over 15% of the total protein synthesis. Northern blot analysis indicated that the increased translation was not a result of an alteration in the ADA mRNA level (data not shown). Rather, the level of translation of the mRNA derived from the transfected plasmid was dramatically stimulated by the presence of VA RNA or by 2-aminopurine. Since both VA RNA and 2-aminopurine may interact with DAI kinase, the results suggest that the level of DAI kinase activity is important in modulating the amount of translation from the ADA mRNA in transfected cells. These results also demonstrate the use of 2-aminopurine as a useful tool to dissect the role of DAI kinase in biological regulation in cells.

To address whether VA RNA or 2-aminopurine has any effect on host protein synthesis or whether the stimulation was specific for plasmid-derived mRNA, it was necessary to

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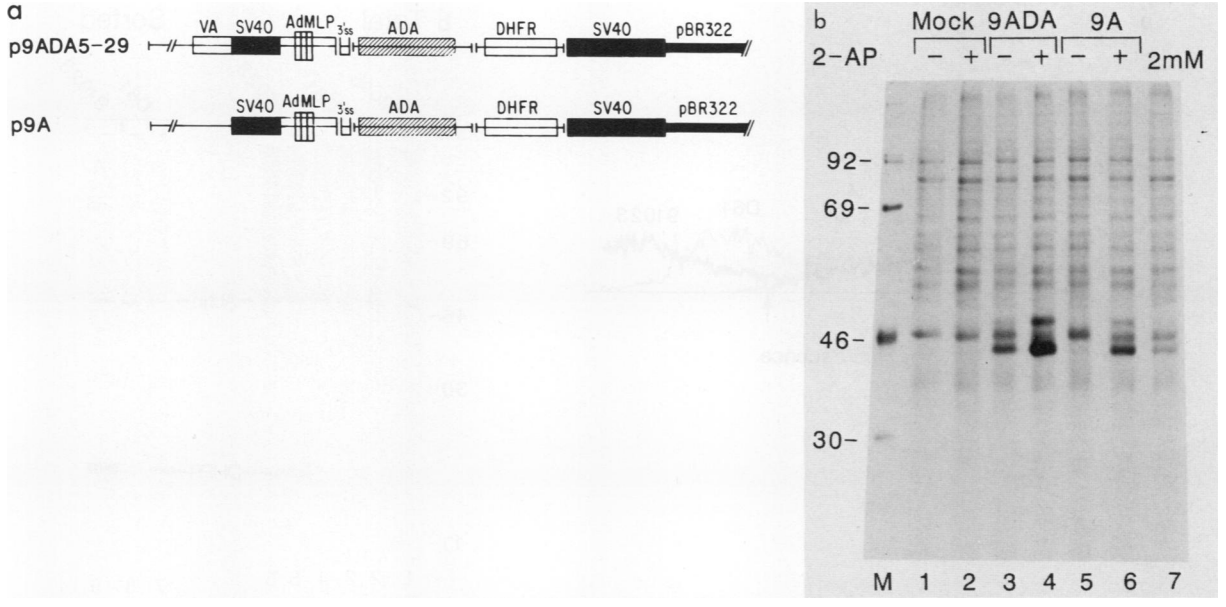

FIG. 1. Effect of VA RNA and 2-aminopurine on total protein synthesis in cells transfected with ADA expression plasmids. (a) The ADA expression plasmids (p9ADA5-29 and p9A) are depicted (30). Both plasmids utilize the adenovirus major late promoter (AdMLP); contain the simian virus 40 (SV40) origin and enhancer element; contain the first, second, and two-thirds of the third leaders of the adenovirus tripartite leader present on adenovirus late mRNAs; and possess an intron of 200 bases (10). The 3′ noncoding region contains a DHFR cDNA and the simian virus 40 early polyadenylation signal. p9A has a deletion of 1.2 kilobases of DNA that encodes adenovirus VA genes I and II. (b) Plasmid DNAs were transfected into COS-1 cells by DEAE-dextran-mediated transfection with the addition of a chloroquine treatment (13, 24). Mock indicates COS cells not transfected with DNA (lanes 1 and 2). At 48 h posttransfection, 10 mM 2-aminopurine (2-AP) was added to the indicated samples, except for one sample transfected with p9A in which 2 mM aminopurine was added (lane 7). After 16 h, cells were labeled with [^{35}S]methionine (100 μCi/ml) for 45 min, and total cell lysates were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described (9). Molecular weight standards are shown (lane M).

study the pattern of protein synthesis of the subset of cells in the population that had received plasmid DNA. To isolate the subpopulation of transfected cells, the cells were transfected with dihydrofolate reductase (DHFR) expression plasmids with or without the VA RNA genes, p91023(B) and pD61, respectively. After transfection, the cells were stained with a fluorescein conjugate of methotrexate, a folate analog which binds DHFR, and labeled in vivo with [^{35}S]methionine. The cells that expressed significantly increased levels of DHFR resulting from expression of the transfected DNA were isolated by fluorescence-activated cell sorting (8). Analysis of cells transfected with p91023(B) and pD61 indicated that approximately 30% of the cells expressed significantly elevated levels of DHFR when compared with cells that had not received DNA (mock in Fig. 2a). Cells that had received the plasmid containing VA RNA (p91023) exhibited approximately 10-fold higher levels of fluorescence than did the cells that had received the VA-deficient plasmid (pD61) (Fig. 2a). However, the fluorescence obtained from either transfected population allowed the convenient isolation of the subpopulation expressing the introduced DHFR gene. Radiolabeled extracts of the total and populations selected by fluorescence-activated cell sorting were analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The pattern of protein synthesis in the original unsorted population demonstrates that the presence of VA RNA or 2-aminopurine resulted in increased translation of the mRNA encoding DHFR (Fig. 2b). Analysis of the pattern of protein synthesis in the isolated transfected subpopulation demonstrates the ratio of synthesis of actin to other endogenous proteins, and the synthesis of actin per unit number of cells was unchanged in the presence or absence of 2-aminopurine or VA RNA (Fig. 2b, lanes 7 to 9). Therefore, the synthesis of endogenous proteins was apparently unaffected by 2-aminopurine or VA RNA. However, DHFR synthesis increased approximately 5- and 11-fold by comparison with the level of actin synthesis in cells treated with 2-aminopurine (Fig. 2b, lanes 7 and 8) or in the presence of VA RNA (Fig. 2b, lanes 8 and 9), respectively. These results demonstrate dramatic specificity of the translational potentiation which was restricted to the plasmid-encoded mRNA. Results of RNA blot hybridization demonstrate that the differences in DHFR synthesis in the presence and absence of VA RNA and 2-aminopurine were not the result of differences in mRNA levels (Fig. 3).

The present results demonstrate that the increase in translation observed with 2-aminopurine and VA RNA was specific to the DHFR mRNA that was encoded by the introduced plasmid. No effect was detected on host mRNAs. This indicates that the plasmid-encoded mRNA is unique in some regard relative to the host mRNAs. One explanation for the specificity of this enhanced translation by VA RNA proposes sequence-specific interactions of VA RNA with mRNA. However, to date, a number of different mRNAs containing different 5′ and 3′ untranslated sequences have exhibited VA stimulation of translation in transfection experiments (7, 25, 26). In addition, 2-aminopurine would not be expected to demonstrate sequence specificity. Alternatively, the results with 2-aminopurine may suggest that the reduced efficiency of translation of the mRNA derived from the plasmid results from a localized activation of DAI kinase in the vicinity of the affected mRNA. One possibility is that dsRNA, generated from asymmetric transcription of the

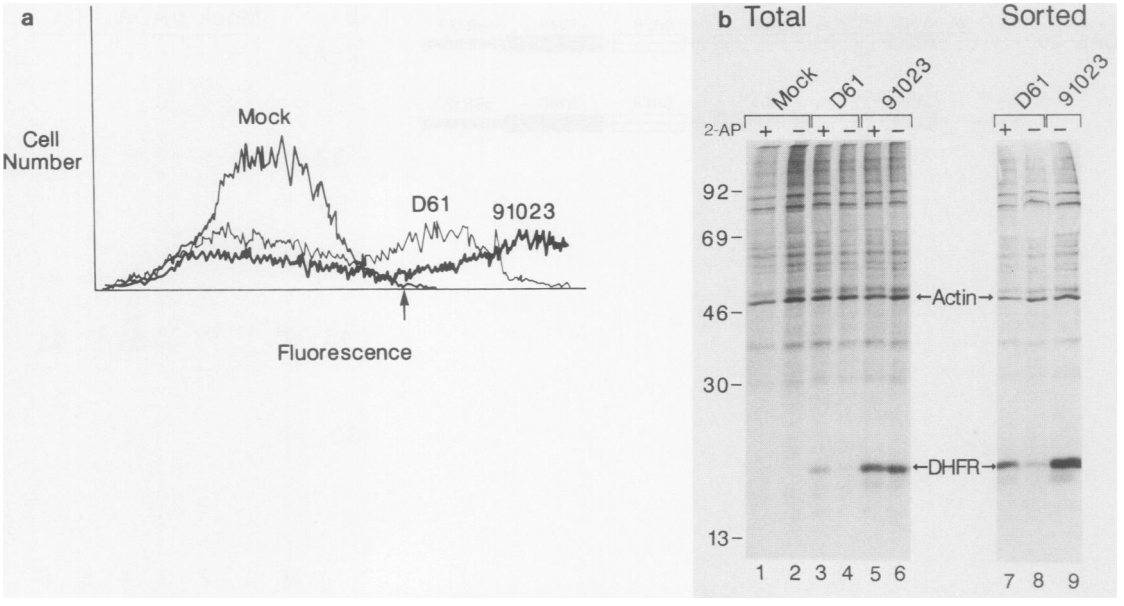

FIG. 2. Total protein synthesis in COS cells transfected with DHFR expression plasmids in the presence and absence of VA RNA and 2-aminopurine. The DHFR expression plasmids pD61 (VA deficient) and p91023(B) (VA containing) have been described previously (7, 29). COS cells were transfected and at 60 h posttransfection were labeled with [^{35}S]methionine for 1 h. Mock indicates cells not transfected with DNA. Total cell extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To isolate the transfected subpopulation, fluorescein-methotrexate was added at 1 μM to the medium 24 h before the cells were labeled with [^{35}S]methionine. (a) Analysis of cells transfected with p91023(B) and pD61. The fluorescence scale represents 5 logs. Positively fluorescent cells (10^5) (arrow indicates the fluorescent gate) were isolated with an EPICS V fluorescent cell sorter. (b) Cell extracts were prepared and analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 5 to 12% polyacrylamide gradient gel. Protein concentrations of the cell lysates were determined by the Bio-Rad protein assay. 2-Aminopurine (10 mM) was added where indicated at 10 h before [^{35}S]methionine labeling. The positions of actin and DHFR migration and the positions of the molecular weight markers are indicated. Addition of 2-aminopurine (2-AP) is indicated. Band intensities were quantitated with an LKB 2202 Ultroscan laser densitometer. DHFR represents 28% of the total protein synthesis in lane 9.

plasmid DNA, is associated with the plasmid-derived mRNA and directly activates DAI kinase. Thus, the activated kinase would be bound specifically to the mRNA containing dsRNA features, resulting in specific, localized depletion of eIF-2. This explanation is consistent with the results of De Benedetti and Baglioni (4), which demonstrate the specific inability to initiate translation of vesicular stomatitis virus mRNA containing a poly(U) tail hybridized to the poly(A) tail. However, alternate explanations, such as the channeling of mRNAs to proper intracellular compartments (1) or the existence of different ribonucleoprotein particles resulting from transcription complexes associated with episomally as opposed to chromosomally localized genes, cannot be ruled out to account for the restricted translational specificity. Experiments are being conducted to test these possibilities.

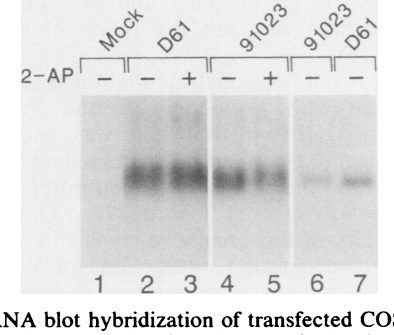

FIG. 3. RNA blot hybridization of transfected COS cells. RNA blot hybridization analysis of total RNA isolated (2) from cells transfected, treated with 2-aminopurine, and harvested at 64 h posttransfection, as described in the legend to Fig. 2. Gel electrophoresis and RNA transfer to nitrocellulose were performed (5, 28), and the blot was hybridized to a nick-translated DHFR cDNA insert. COS cells not transfected with DNA are indicated as mock (lane 1). Results from separate transfection and RNA blot hybridization experiments are shown (lanes 1 to 5 and lanes 6 and 7). Addition of 2-aminopurine (2-AP) is indicated.

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LITERATURE CITED

1. **Blobel, G.** 1985. Gene gating: a hypothesis. Proc. Natl. Acad. Sci. USA **82**:8527–8529.
2. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
3. **De Benedetti, A., and C. Baglioni.** 1983. Phosphorylation of initiation factor eIF-2α, binding of mRNA to 48 S complexes, and its reutilization in initiation of protein synthesis. J. Biol. Chem. **258**:14556–14562.
4. **De Benedetti, A., and C. Baglioni.** 1984. Inhibition of mRNA binding to ribosomes by localized actiation of dsRNA-dependent protein kinase. Nature (London) **311**:79–81.
5. **Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. R. Darnell, Jr.** 1981. Transcriptional control in the production of liver-specific mRNAs. Cell **23**:731–739.
6. **Jagus, R., W. F. Anderson, and B. Safer.** 1981. The regulation of initiation of mammalian protein synthesis. Prog. Nucleic Acid Res. Mol. Biol. **25**:127–185.

7. **Kaufman, R. J.** 1985. Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. Proc. Natl. Acad. Sci. USA **82:** 689–693.
8. **Kaufman, R. J., J. R. Bertino, and R. T. Schimke.** 1978. Quantitation of dihydrofolate reductase in individual parental and methotrexate-resistant murine cells. J. Biol. Chem. **253:**5852–5860.
9. **Kaufman, R. J., P. Murtha, D. E. Ingolia, C.-Y. Yeung, and R. E. Kellems.** 1986. Selection and amplification of heterologous genes encoding adenosine deaminase in mammalian cells. Proc. Natl. Acad. Sci. USA **83:**3136–3140.
10. **Kaufman, R. J., and P. A. Sharp.** 1982. Construction of a modular dihydrofolate reductase cDNA gene: analysis of signals utilized for efficient expression. Mol. Cell. Biol. **2:**1304–1319.
11. **Kitajewski, J., R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, B. Thimmappaya, and T. Shenk.** 1986. Adenovirus VAI RNA antagonizes the antiviral action of interferon by preventing activation of the interferon-induced eIF2 alpha kinase. Cell **45:**195–200.
12. **Lebleu, B., G. C. Sen, S. Shaila, B. Cabrer, and P. Lengrel.** 1976. Interferon, double-stranded RNA and protein phosphorylation. Proc. Natl. Acad. Sci. USA **73:**3107–3111.
13. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11:**1295–1308.
14. **Mathews, M. B. (ed.).** 1986. Translation control. Current communications in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Ochoa, S.** 1983. Regulation of protein synthesis in eukaryotes. Arch. Biochem. Biophys. **223:**325–349.
16. **O'Malley, R. P., T. M. Mariano, J. Siekierka, and M. B. Mathews.** 1986. A mechanism for the control of protein synthesis by adenovirus VA RNA_I. Cell **44:**391–400.
17. **Reichel, P. A., W. C. Merrick, J. Siekierka, and M. Mathews.** 1985. Regulation of a protein synthesis initiation factor by adenovirus associated RNA I. Nature (London) **313:**196–200.
18. **Roberts, W. K., A. Hovanessian, R. E. Brown, M. J. Clemens, and I. M. Kerr.** 1976. Interferon-mediated protein kinase and low molecular-weight inhibitor of protein synthesis. Nature (London) **264:**477–480.
19. **Safer, B.** 1983. 2B or not 2B: regulation of the catalytic utilization of eIF-2. Cell **33:**7–8.
20. **Samuel, C. E., D. A. Farris, and D. A. Eppstein.** 1977. Mechanism of interferon action. Kinetics of interferon action in mouse L_{929} cells: translational inhibition, protein phosphorylation, and mRNA phosphorylation and degradation. Virology **83:**56–71.
21. **Schneider, R. J., B. Safer, S. M. Munemitsu, C. E. Samuel, and T. Shenk.** 1985. Adenovirus VA RNA_I prevents phosphorylation of the eukaryotic initiation factor 2 subunit subsequent to infection. Proc. Natl. Acad. Sci. USA **82:**4321–4325.
22. **Schneider, R. J., C. Weinberger, and T. Shenk.** 1984. Adenovirus VAI RNA facilitated the initiation of translation in virus-infected cells. Cell **37:**291–298.
23. **Siekierka, J., T. M. Mariano, P. A. Reichel, and M. B. Mathews.** 1985. Translational control by adenovirus: lack of virus associated RNA_I during adenovirus infection results in phosphorylation of initiation factor eIF-2 and inhibition of protein synthesis. Proc. Natl. Acad. Sci. USA **82:**1959–1963.
24. **Sompayrac, L. M., and K. J. Dana.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78:**7575–7578.
25. **Svensson, C., and G. Akusjärvi.** 1984. Adenovirus VA RNA_I: a positive regulator of mRNA translation. Mol. Cell. Biol. **4:**736–742.
26. **Svensson, C., and G. Akusjärvi.** 1985. Adenovirus VA RNA_I mediates a translational stimulation which is not restricted to the viral mRNAs. EMBO J. **4:**957–964.
27. **Thimmappaya, B., C. Weinberger, R. J. Schneider, and T. Shenk.** 1982. Adenovirus VA RNA_I is required for efficient translation of viral mRNA at late times after infection. Cell **31:**543–551.
28. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.
29. **Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, D. P. Lexenberg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. S. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang, and S. C. Clark.** 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science **228:**810–815.
30. **Yeung, C.-Y., D. E. Ingolia, D. B. Roth, C. Shoemaker, M. R. Al-Ubaili, J.-Y. Yen, C. Ching, C. Bobonis, R. J. Kaufman, and R. E. Kellems.** 1985. Identification of functional murine adenosine deaminase cDNA clones by complementation in Escherichia coli. J. Biol. Chem. **260:**10229–10307.
31. **Zilberstein, A., P. Federmann, L. Schulman, and M. Revel.** 1976. Specific phosphorylation of a pretein associated with ribosomes of interferon-treated mouse L cells. FEBS Lett. **68:**119–124.