Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1258–1262, April 1975

Selective Blockage of Initiation of Host Protein Synthesis in RNA-Virus-Infected Cells

(initiation of protein synthesis/differential inhibition/virus-induced suppression)

D. L. NUSS, H. OPPERMANN, AND G. KOCH

Roche Institute of Molecular Biology, Department of Cell Biology, Nutley, New Jersey 07110

Communicated by Severo Ochoa, January 13, 1975

ABSTRACT **Poliovirus mRNA and mRNA transcribed from vesicular stomatitis virus and reovirus genomes efficiently direct protein synthesis *in vivo* under experimental conditions where the initiation of host protein synthesis is selectively blocked. The selective blockage of host peptide chain initiation after exposure to hypertonic medium indicates that the translation of viral mRNA is more efficiently initiated than is the translation of host mRNA. It further suggests that virus-directed suppression of host protein synthesis could proceed by a mechanism involving a nonspecific decrease in the rate of peptide chain initiation. Exposure of infected cells to hypertonic medium provides a unique tool with which to study early events in the infectious cycle by permitting the efficient unmasking of virus-specific polypeptide synthesis.**

Infection of cultured mammalian cells by a number of RNA-containing animal viruses results in the efficient suppression of host protein synthesis (1–8). Previous studies with picornavirus (1, 3, 5, 9–11) and vesicular stomatitis virus (VSV) (6) implicate a viral protein as responsible for virus-directed suppression of host protein synthesis. Although recent evidence suggests that some virus-specific peptides bind to cellular ribosomes (12, 13), the identification of a protein factor which can selectively suppress the translation of host mRNA *in vitro* has not been forthcoming (3, 4). In plasmacytoma cell-free preparations, the addition of encephalomyocarditis virus RNA results in the complete suppression of cellular mRNA translation (4). Thus, competition between viral and host mRNA for available ribosomes may also play an important role in suppressing host protein synthesis.

Saborio *et al.* (14), reported that the initiation of protein synthesis could be selectively and reversibly inhibited in HeLa cells and in poliovirus-infected HeLa cells by increasing the osmolarity of the growth medium. This inhibition of peptide chain initiation is independent of the solute (NaCl, KCl, NH_4Cl, or sucrose) used to increase the osmolarity of the medium. Under these experimental conditions, peptide chain elongation, termination, and processing proceed unaffected, resulting in a complete breakdown of polyribosomes. However, a higher osmolarity is required to completely inhibit peptide chain initiation in poliovirus-infected HeLa cells than in uninfected cells (14).

Inhibition of protein synthesis by excess NaCl and, apparently, virus-directed inhibition of host protein synthesis both act at the level of peptide chain initiation (14, 15), and in both cases the protein synthesizing machinery remains functional (14, 15). Thus, inhibition by hypertonic medium provides a useful tool with which to ask basic questions related to the process of virus-directed suppression of cellular protein synthesis.

Abbreviations: VSV, vesicular stomatitis virus; BHK, baby hamster kidney cells; MEM, minimal essential medium.

MATERIALS AND METHODS

Growth of Cells and Infection with Virus. Baby hamster kidney (BHK) cells were grown in Joklik-modified minimal essential medium (MEM) (Gibco F-13) supplemented with 5% fetal calf serum plus nonessential amino acids, and a double complement of vitamins. Cultures were maintained at densities of between 5 and 12 $\times$ 10^5 cells per ml. HeLa S_3 cells were grown as previously described (14).

Adsorption of poliovirus Type 1, strain Mahoney (16), and VSV, serotype Indiana (17), was allowed to occur at room temperature for 20 min with HeLa cells suspended at a density of 1 $\times$ 10^7 cells per ml in serum-free MEM. Following adsorption, the cell suspensions were diluted to a density of 1 $\times$ 10^6 cells per ml with MEM medium containing 5% fetal calf serum and incubated at 37°. Adsorption of reovirus, Type 3, kindly provided by A. J. Shatkin, Roche Institute of Molecular Biology, to BHK cells was performed at 37° for 30 min. In all experiments the time of dilution of virus adsorbed cells was taken as time zero of the infectious cycle.

Incorporation Studies. The incorporation of [^{3}H]leucine (5 Ci/mmol) (New England Nuclear) and [^{35}S]methionine (330 Ci/mmol) (New England Nuclear) into cellular protein was determined according to the method of Mans and Novelli (18). [^{3}H]Uridine (New England Nuclear) incorporation into RNA was determined according to the method of Trown and Bilello (19).

Analysis of Cytoplasmic Extracts by Polyacrylamide/Sodium Dodecyl Sulfate Gel Electrophoresis. Cytoplasmic extracts were prepared as previously described (14) and analyzed by polyacrylamide gel electrophoresis as described by either Maizel (20) or Laemmli (21).

RESULTS

Time-dependent increase in resistance of protein synthesis to inhibition by hypertonic medium in virus-infected cells

At appropriate times after infection, cells were transferred to hypertonic medium (isotonic medium is 110 mM in NaCl) for 15 min, time sufficient for complete run-off of ribosomes from messengers whose translation has been blocked (14), and then were pulse labeled with [^{3}H]leucine and [^{35}S]methionine. Fig. 1A, lower panel, shows the increase in resistance to excess NaCl inhibition in HeLa cells after poliovirus infection. At final NaCl concentrations of 210, 230, and 250 mM, the resistance to excess NaCl inhibition is seen to increase from values of 7–2% at 30-min post-infection to values of 80%, 65%, and

40%, respectively by 3 hr after infection. The decline in resistance observed after 3 hr is thought to be a result of cytotoxic effects of poliovirus infection. Mock-infected cells showed little change in either the total rate of amino-acid incorporation or in the percent resistance to inhibition by hypertonic medium over the incubation period. Fig. 1B, lower panel, shows similar results obtained from VSV-infected HeLa cells. The resistance reaches 54%, 37%, and 25% at the respective NaCl concentrations by approximately 4 hr post-infection. No decrease in resistance is observed by as late as 7-hr post-infection in VSV-infected cells. Fig. 1C, lower panel, shows the increase in resistance at two excess salt concentrations (210 mM and 230 mM) with time after infection of BHK cells by reovirus. The overall kinetics of the increase in resistance, relative to the length of the infectious cycle, are similar in all three virus-infected cells.

As illustrated in Panel C, uninfected BHK cells showed slightly more resistance to excess NaCl inhibition than HeLa cells. Although the resistance of BHK cells varied somewhat, depending on incubation conditions, the cell density, and the nutritional state of the cells, the maximum difference between the resistance of infected and mock-infected cells to inhibition by 100 mM excess NaCl, the difference between 45% and 15% in Fig. 1 Panel C, was routinely 2.5- to 3-fold in reovirus-infected BHK cells. In poliovirus-infected HeLa cells this value was usually 10-fold, while in VSV-infected HeLa cells it was 6- to 8-fold.

Also shown in Fig. 1 is the total amino acid incorporation observed during the 15-min pulses performed after infection by each virus. In poliovirus-infected HeLa cells, Fig. 1A, upper panel, the rate of protein synthesis is shown to rapidly decline by approximately 75% by 2 hr post-infection. An increase in the rate is observed from 2 to 3 hr due to the contribution of viral protein synthesis but the rate again declines by 4 hr. Notice that mock-infected cells also show a 10% decrease in the rate of amino-acid incorporation by 4 hr after time of dilution. In VSV-infected cells, Fig. 1B, upper panel, the rate of total protein synthesis is inhibited by approximately 50% by 7 hr of infection, while in mock-infection the decline is 20% over the same period. By 2- to 3-hr post-infection, however, host-specific protein synthesis is almost completely inhibited, as will be shown in a subsequent figure. In reovirus-infected BHK cells, Fig. 1C, upper panel, the rate of amino-acid incorporation increases to its highest level by 7-hr post-infection and then declines after 11 hr. Mock-infected cells also show a similar increase in the rate of protein synthesis, but no drop is observed by 13 hr after dilution. This increase in the rate of amino-acid incorporation, in part, represents a recovery phase after incubation of BHK cells at a density of 1×10^7 cells per ml at 37° for 30 min during virus adsorption. If mock-infected BHK cells are incubated at this cell concentration at room temperature for 15 min, this effect is not observed.

Fig. 2 shows, again, the decrease in total protein synthesis, the increase in protein synthesis resistant to excess NaCl inhibition with time after infection of HeLa cells by poliovirus, and the relationship to viral RNA synthesis. The kinetics of viral RNA synthesis coincides with, or slightly precedes, the increase in protein synthesis resistant to inhibition by excess NaCl, with both curves reaching a peak between 180- and 240-min post-infection. The temporal relationship between viral RNA synthesis and resistance to inhibition by hypertonic medium suggests that the two events are directly linked.

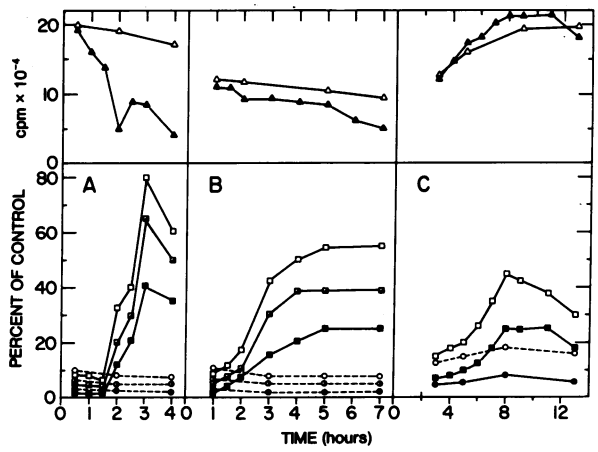

FIG. 1. Time-dependent increase in resistance of protein synthesis to excess-NaCl-mediated inhibition in infected cells. Virus infection was performed as described in the *Materials and Methods* section. Cells were collected by centrifugation and resuspended at a density of 4×10^6 cells per ml in MEM-25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid containing $^1/_{20}$ the normal complement of methionine and leucine. The NaCl concentration of the MEM medium, normally 110 mM, was adjusted and, following 15 min of incubation at 37°, 2.5 μCi of [^{35}S]methionine and 7.5 μCi of [^{3}H]leucine were added. Following a 15-min incubation, the incorporation of labeled amino acids into protein was determined as indicated in the *Materials and Methods* section. The data are presented as [the amount of radioactivity (^{35}S and ^{3}H), measured in cpm, incorporated in the NaCl-treated cells] ÷ [the radioactivity incorporated in the untreated cells] × 100. Squares represent percent resistance of infected cells while resistance in mock-infected cells is designated by the circles. The final NaCl concentration are 210 mM (□ and ○), 230 mM (◪ and ◒), and 250 mM (■ and ●). Panel A = HeLa cells infected with 30 plaque forming units (PFU) poliovirus per cell; Panel B = HeLa cells infected with 10 PFU VSV per cell; Panel C = BHK cells infected with 60 PFU reovirus per cell. Upper panels show the total amino acid incorporation in infected (▲——▲) and mock-infected (△——△) cells pulse labeled in isotonic medium during the corresponding 15-min pulse represented in the lower panels.

Amplification of virus-directed suppression of host protein synthesis by hypertonic medium

If the translation of virus-specific mRNA but not host mRNA is resistant to inhibition by excess NaCl, then the peptides labeled during a 15-min pulse 15 min after NaCl addition, even at early times in the infectious cycle, should be predominantly virus specific.

Fig. 3 shows the polyacrylamide-sodium dodecyl sulfate gel profiles of cytoplasmic extracts from VSV-infected HeLa cells pulse labeled as described above, in the presence or absence of 100 mM excess NaCl at different times in the infectious cycle. Panel A shows the profile obtained from mock-infected cells while Panels B, C, and D are from infected cells pulse labeled at 90, 135, and 240 min post-infection, respectively.

These gel profiles show that a 15-min block with excess NaCl selectively inhibits host protein synthesis, thereby potentiating the normal course of virus-directed suppression of host protein synthesis. At 90-min post-infection, the percentage of labeled peptides that are virus-specific increased from less than 15% in the control cells to greater than 65% in cells exposed to hypertonic medium. Panel C shows that at

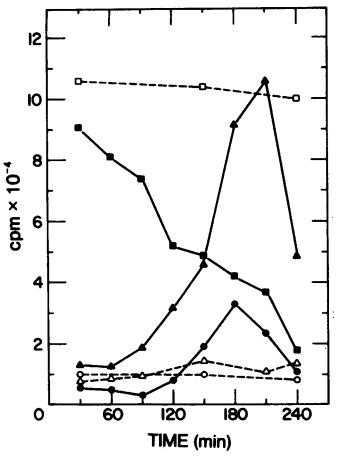

FIG. 2. Relationship between total protein synthesis, viral RNA synthesis, and resistance of protein synthesis to inhibition mediated by excess NaCl. HeLa cells were infected by poliovirus at a multiplicity of 10 PFU per cell as described in the *Materials and Methods* section. The infected culture was split at the time of dilution and amino acid incorporation and NaCl resistance (210 mM NaCl) measured, as described in Fig. 1, in half of the culture. To the other half of the culture was added 5 μg/ml of actinomycin D. At the appropriate times, 0.3 ml aliquots of cells were pulse labeled for 15 min with 10 μCi of [^{3}H]uridine. The incorporation of [^{3}H]uridine into RNA was measured as described in ref. 19. This figure shows total amino acid incorporation (^{35}S + ^{3}H) in mock-infected cells (□- - -□) and infected cells (■——■) pulse labeled in isotonic (110 mM NaCl) medium; amino-acid incorporation in mock-infected (○- - -○) and infected cells (●——●) pulse labeled in hypertonic (210 mM NaCl) medium; and [^{3}H]uridine incorporation in mock-infected (△- - -△) and infected cells (▲——▲) treated with actinomycin D.

135-min post-infection, this value increases from approximately 35% to greater than 85%. It should be mentioned that in the infected cell in which the percentage of viral peptide synthesis is only 10% of the total protein synthesis, if host protein synthesis is selectively inhibited by 95%, peptides labeled during a 15-min pulse would still be expected to consist of at least 30% host-specific peptides.

Two additional points are evident from Fig. 3. A peptide or peptides migrating at approximately fraction 82, designated HP-20 (HeLa protein molecular weight 20,000), of the mock-infected NaCl-treated profile (Panel A) is somewhat resistant to NaCl inhibition. This same peak is evident in Panels B, C, and D, showing that HP-20 is partially resistant both to inhibition by excess NaCl and to virus-directed shut off. Whereas the relative synthesis of poliovirus-specific peptides, which are translated from a polycistronic mRNA with one initiation site (14, 22), showed no change with increasing NaCl concentrations (data not shown), the relative amounts of the five VSV peptides synthesized during a 15-min pulse is influenced by the medium hypertonicity, as most clearly shown in Panel D. The relative amounts of N, NS, and L peptides is increased with respect to the amounts of the M and G peptides. It is clear that when this method is used to unmask virus-specific proteins, especially for quantitative studies, the effect of NaCl on the translation of each viral peptide must be considered.

Fig. 4 shows gel profiles obtained from poliovirus-infected HeLa cells at 105 min after infection (Panel C) and from mock-infected HeLa cells (Panel A). Poliovirus-specific peptides are impossible to identify in the gel profile obtained from extracts of untreated cells, but are prominent in the gel profile obtained from cytoplasmic extracts from cells pulse labeled in hypertonic medium (Panel C). Again, a resistant peak, HP-20, is observed in profiles of both infected and mock-infected cell extracts, migrating at fraction 64 in this gel system. Panels B and D show the gel profiles obtained from mock-infected and reovirus-infected BHK cells, respectively. The reovirus-encoded peptide classes λ, μ, and σ (27) are prominent in lysates of cells pulse labeled in hypertonic medium (Panel D).

DISCUSSION

In poliovirus-, VSV-, or reovirus-infected cells, the mRNA species that are translated after exposure of cells to hypertonic medium are predominantly viral mRNA (Fig. 3 and 4). These results indicate that the observed increase in resistance to inhibition by excess NaCl after virus infection (Fig. 1) is a reflection of an intrinsic resistance in the translation of virus-specific mRNA to inhibition by hypertonic medium and not due to general effects resulting from viral infection. If general effects, such as changes in membrane permeability, were responsible for the results shown in Fig. 1, the translation of all populations of mRNA should be equally affected by exposure to hypertonic medium.

Although the exact mechanism whereby exposure of cells to excess NaCl results in the inhibition of peptide chain initiation is not clear, one straight-forward explanation is that it lowers the rate of initiation by altering the affinity between ribosomes and mRNA. It has previously been suggested that protein synthesis in animal cells can be regulated by membrane-mediated events (23, 24). This view is supported by the observation that several inhibitors of *in vivo* peptide chain initiation such as increased medium osmolarity (14), L-1-tosylamido-2-phenylethyl chloromethyl ketone (23), and cytochalasin B (25), show little effect on initiation in cell-free extracts prepared from HeLa cells or L cells (manuscript in preparation). Consequently, the experimental results reported in this paper cannot readily be tested in presently available cell-free systems.

The selective blockage of host peptide chain initiation after exposure of virus-infected cells to hypertonic medium indicates that the initiation of viral mRNA translation is more efficient than that of host mRNA. That is, viral mRNA possesses a stronger affinity for ribosomes, or a higher rate of formation of initiation complexes, than does host mRNA. This interpretation could readily explain why infection of cells by isolated viral mRNA is promoted under experimental conditions that interfere with cellular protein synthesis (26). Competition between viral and host mRNA for ribosomes is likely to have an impact on host protein synthesis and may be an adequate explanation for the degree of inhibition of host protein synthesis observed in reovirus-infected cells (Fig. 4D, and ref. 27). The possible role of such competition in encephalomyocarditis-virus-infected myeloma cells (4) and Semliki Forest virus-infected BHK cells (28) has recently been discussed.

Figs. 1 and 2 show that in poliovirus-infected HeLa cells host protein synthesis is significantly inhibited at times prior to the synthesis of significant levels of viral protein and RNA, indicating that an additional mechanism is responsible for this inhibition. Existing data suggest that a virus-specific or virus-induced factor is involved in host protein suppression

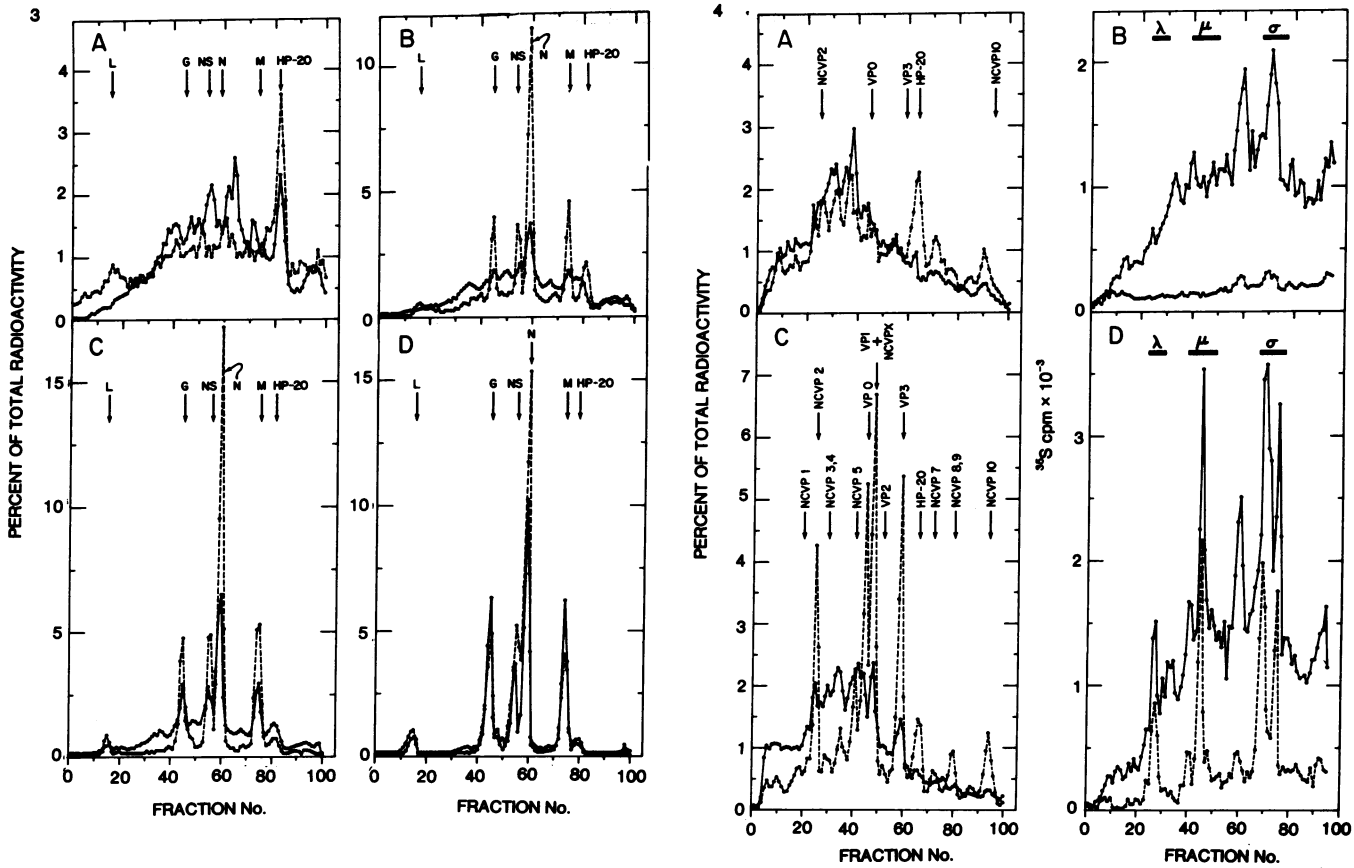

FIG. 3. Polyacrylamide/dodecyl sulfate gel electrophoresis of cytoplasmic extracts from VSV-infected HeLa cells pulse labeled in hypertonic medium. VSV infected HeLa cells (4.75 ml) (4×10^6 cells per ml) were treated with 100 mM excess NaCl for 15 min and then pulse labeled with 25 μCi of [^{35}S]methionine and 50 μCi of [^{3}H]leucine for 15 min. After removal of a 50 μl aliquot for the determination of hot-acid-precipitable radioactive material, each sample was diluted with 10 volumes of semi-frozen serum-free MEM containing a large excess of unlabeled methionine and leucine, centrifuged, washed with the same medium, centrifuged, and resuspended in warm serum-supplemented MEM containing excess methionine and leucine. Following a 15-min incubation period, cytoplasmic extracts were prepared as indicated in the *Materials and Methods* section. Extracts were analyzed on 10 cm, 10% polyacrylamide/dodecyl sulfate gels according to Laemmli (21). Gel profiles from NaCl-treated (O- - -O) and untreated (●- - -●) mock-infected cells are shown in Panel A. Panels B, C, and D show profiles for NaCl-treated (O- - -O) and untreated (●——●) cells at 90-, 135-, and 240-min post-infection, respectively. The percent resistance, as measured prior to preparation of cytoplasmic extracts, and the total radioactivity recovered from each gel were: Panel A, 6.8%, control = 534,026 cpm, treated = 30,022 cpm; Panel B, 9.7%, control = 372,054 cpm, treated = 37,836 cpm; Panel C, 21.4%, control = 347,620, treated = 84,174 cpm; Panel D, 36.4%, control = 420,809 cpm, treated = 132,974 cpm. The arrows designate the distance of migration of the five virus-encoded peptides N, G, NS, N, and M (17).

FIG. 4. Polyacrylamide/dodecyl sulfate gel electrophoresis of cytoplasmic extract from poliovirus-infected HeLa cells and reovirus-infected BHK cells. This experiment was performed essentially as described in Fig. 3 except that NaCl-treated (100 mM excess) poliovirus-infected and mock-infected cells were pulsed at 90-min post-infection with 70 μCi of [^{35}S]methionine and 130 μCi of [^{3}H]leucine, while untreated cells were pulsed with 25 μCi of [^{35}S]methionine and 65 μCi of [^{3}H]leucine. Following the 15-min pulse period the cells were incubated in medium containing excess methionine and leucine for 30 min. In reovirus-infected and mock-infected BHK cells, NaCl-treated (110 mM excess) cells were pulsed at 6-hr post-infection with 37.5 μCi of [^{35}S]methionine while untreated cells received 7.5 μCi of [^{35}S]methionine. The chase period was 15 min. Panel A: mock-infected HeLa cells, treated (O- - -O) 30,939 cpm per gel and untreated (●——●) 257,831 cpm per gel. Percent resistance was 6.0%. Panel C: poliovirus-infected cells, treated (O- - -O) 53,059 cpm per gel and untreated (●——●) 212,277 cpm per gel. Percent resistance was 13.4%. Panel C: mock-infected BHK cells, treated (O- - -O) 16,960 cpm per gel and untreated (●——●) 88,413 cpm per gel. Percent resistance was 8%. Panel D: reovirus-infected BHK cells, treated (O- - -O) 34,555 cpm per gel, and untreated (●——●) 117,980 cpm per gel. Percent resistance was 14.4%. Extracts from poliovirus-infected cells were analyzed according to Maizel (22), while extracts from reovirus-infected cells were analyzed according to Laemmli (21). The arrows in panel A and B indicate the distance of migration of the poliovirus capsid (VP0-4) and noncapsid (NCVP1-10) polypeptides (14). The bars in Panels B and D indicate the migration distance of the reovirus-encoded polypeptide classes λ, μ, and σ (27). Similar results are obtained whether individual gels receive the same amount of protein or the same amount of radioactivity.

in poliovirus- and VSV-infected cells (1, 3, 5, 6, 9–11), although the actual existence of such a factor has not been demonstrated (3, 4).

The observation that the selective inhibition of peptide chain initiation by an inhibitor such as excess NaCl can result in the potentiation of virus-directed suppression of host protein synthesis at early times in the infectious cycle suggests that the proposed suppression factor(s) could also act at the level of peptide chain initiation as previously postulated (15). This proposed factor(s) need not possess the capacity to actively discriminate between viral and host mRNA, but could perform its function by indiscriminately lowering the rate of

peptide chain initiation for the translation of all mRNA. Such an alteration of the rate constant for initiation complex formation would permit the translation of mRNA that possesses a strong affinity for ribosomes, such as viral mRNA, while the translation of mRNA with a lower affinity, host mRNA, would be suppressed. A similar mechanism has recently been suggested to explain the relative synthesis of α and β chains of globin in the rabbit reticulocyte system under several different experimental conditions (29).

It remains to be investigated how the postulated factor could alter the rate of initiation complex formation. Furthermore, it is not known whether the factor is a protein coded for by the viral genome, or whether virus infection might trigger normal host control mechanism to inhibit host protein synthesis.

Further studies are called for to determine whether inhibition of protein synthesis by excess NaCl and by virus infection act at the same or different steps in peptide chain initiation. In any event, inhibition of protein synthesis by excess NaCl provides a unique tool with which to further elucidate the process of virus-directed suppression of host protein synthesis. This method will also make possible a study of events early in the virus infections cycle by permitting the efficient unmasking of virus-specific peptide synthesis.

The initiation of translation of different classes of cellular mRNA in uninfected cells can be differentially inhibited by exposure of cells to increasing concentrations of NaCl (30). Therefore, the use of this method to selectively inhibit peptide chain initiation will also be useful in further elucidating mechanisms governing the control of translation in eukaryotic cells.

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