Rates of Initiation of Protein Synthesis by Two Purified Species of Vesicular Stomatitis Virus Messenger RNA*

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Two vesicular stomatitis virus (VSV) messenger RNAs, those encoding the N (M₀ = 52,500) and G (M₀ = 63,500) can be isolated in pure form, and the nucleotide sequence of the 5' ends and ribosome binding sites of these mRNAs have been determined. Thus, this is a suitable eukaryotic system in which to study structure-function relationships in mRNA. Our present results indicate that, under conditions which obtain in infected cells or in the reticulocyte cell-free system, the rates of polypeptide chain initiation and elongation of these two mRNAs is the same: (a) the molar ratio of G to N protein made in infected cells is very similar to the molar ratio of G to N mRNAs recovered from these cells, as was shown previously by Villarreal et al. ([1976] Biochemistry 15, 1882-1888); (b) in the infected cell, the average size of polyribosomes synthesizing G protein is about 20% larger than that synthesizing N. Thus, the density of ribosomes on the translated part of each mRNA seems to be the same; (c) the rate of binding of G and N mRNAs to reticulocyte ribosomes and formation of 80 S initiation complexes is the same; (d) the steady state size of reticulocyte polysomes containing G and N mRNAs, and the rate at which these polysomes are formed in vitro, is the same.

However, two types of experiments show that, under certain conditions of inhibition of the overall rate of chain initiation, G mRNA is translated up to 2-fold less efficiently than is N mRNA. First, as was shown by Nuss and Koch ([1976] J. Virol. 17, 283-286), treatment of infected cells with media containing hypertonic salt results in inhibition of synthesis of all VSV proteins, but synthesis of G is reduced twice as much as is N. Second, we showed that addition to a wheat germ protein synthesis reaction of the inhibitors of polypeptide chain initiation poly(dT) or aurintricarboxylic acid results in inhibition of initiation of translation on all VSV mRNAs, but that synthesis of G is inhibited twice as much as is N. We conclude that G and N mRNAs do differ in their affinity toward or requirement for some component or factor required for chain initiation, possibly that substance(s) whose function is blocked by the above inhibitors. We postulate that the amount of this substance is not normally rate-limiting for mRNA translation, and thus that G and N mRNAs are normally translated at the same efficiency.

Different messenger RNAs differ in the rates of attachment to ribosomal subunits or in other stages of initiation of protein synthesis (1-3). For example, in the rabbit reticulocyte each molecule of α-globin mRNA initiates protein synthesis only 60% as frequently as does each β-globin mRNA (1, 4-6). In prokaryotes, extreme variations in initiation rates have been observed. The rates of initiation of translation of the three cistrons of bacteriophage f2 RNA differ more than 20-fold (7), and a similar situation obtains for several cistrons in bacteriophage λ mRNA (8).

Vesicular stomatitis virus is a suitable eukaryotic system in which to study structure-function relationships of messenger RNA. VSV infection results in synthesis of only five VSV mRNAs, each encoding a single VSV polypeptide (reviewed in Ref. 9). Two VSV mRNAs can be isolated in pure form by polyacrylamide gel electrophoresis; those encoding the N (M₀ = 32,500) and G (M₀ = 63,500) proteins (10-12). The mRNAs encoding the NS (M₀ = 37,000) and M (M₀ = 35,000) proteins are not separable, and the mRNA encoding the largest VSV protein (L, M₀ = 180,000) has not been completely purified. All VSV mRNAs contain at the 5'-end the same sequence of nucleotides: m⁵G(5')ppp(5')tmAmpA-(mp)CpApGp... (13); also, the sequence of the ribosome binding sites of the G, N, and NS mRNAs have been determined (14).

Two studies suggest that all VSV mRNAs initiate protein synthesis at the same relative rate in the infected cell. In one, the relative synthesis of the VSV proteins and mRNAs was compared (15), but this type of study is subject to many uncertainties: All of the mRNA encoding the G protein is bound to intracellular membranes (10, 16, 18) and the extent of recovery of this mRNA from cells is uncertain. In addition, there is uncertainty in the molecular weights of certain VSV proteins and mRNAs (9, 12, 15), so it is difficult, in any case, to compare the molar amount of mRNA with the molar amount of protein produced. In the second study (18), the relative synthesis of VSV proteins was measured in the presence of inhibitors of polypeptide chain elongation, but

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1 The abbreviations used are: VSV, vesicular stomatitis virus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATA, aurintricarboxylic acid; EMC, encephalomyocarditis.
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this assay for relative initiation frequency by different mRNAs is subject to the untested assumptions that treatment with the inhibitors saturates the mRNAs with ribosomes (41).

Nuss and Koch (19), by contrast, found that synthesis of VSV G and M proteins in the infected cell is much more sensitive to treatment with hypertonic salt solutions than is that of N or NS proteins; this result suggests that VSV G and M mRNAs are less efficient in chain initiation than are N or NS mRNAs (20-23). In this paper we have employed several assays to determine the relative rates of polysome chain initiation on VSV G and N mRNAs. The most direct of these involves the measurement of the sizes of polyribosomes which contain these mRNAs, both in the infected cell and in the reticulocyte cell-free system. We conclude that, under the conditions which occur in the infected cell or in the reticulocyte lysate, G and N mRNAs do initiate synthesis of polyribosomes at the same rate. However, certain treatments which reduce the overall rate of chain initiation do result in preferential inhibition of synthesis of the VSV G protein. This suggests that G mRNA requires for translation more of some component of the initiation machinery than does N mRNA.

MATERIALS AND METHODS

Phosphate Labeling and Fractionation of Infected Cells—A modification of an earlier procedure was used (24, 20). One hundred milliliters of Chinese hamster ovary (CHO) cells in spinner culture at 4 x 10^9 cells/ml were concentrated by centrifugation (1500 x g, 5 min), washed twice in PO_4-free medium, and then resuspended in 10 ml of PO_4-free medium. Actinomycin D (final concentration 5 μg/ml) and VSV (multiplicity of infection, 6) were then added, and incubation at 37° continued. At 30 min an additional 10 ml of PO_4-free medium was added, and at 60 min after addition of virus, 10 μCi of carrier-free 32P0_4 (New England Nuclear Corp., Boston, Mass.). Incubation was continued for an additional 3 h.

To prepare polyomes, emetine (final concentration 100 μg/ml) was added to the cells to block polysome chain elongation and the cultures were chilled on ice. All subsequent steps were done at 4°. Cells were recovered by centrifugation, washed once in phosphate-buffered saline (0.9% NaCl solution) containing 100 μg/ml of emetine, once in Buffer A (0.01 M 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid (Hepes), pH 7.5, 0.01 M KCl, 0.0015 M MgOAc), and resuspended in Buffer A at a concentration of 1.5 x 10^9 cells/ml. After swelling on ice for 10 min, the cells were disrupted with 40 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Nuclei were removed by centrifugation for 4 min at 4000 x g. To 1.5 ml of postnuclear supernatant was added 1.0 ml of Buffer B (0.12 M Tris, pH 8.5, 0.24 M NaCl, 0.0075 M MgOAc), then 0.3 ml of a 10% solution of sodium deoxycholate, pH 8.5. Following a 2-s mixing, 0.3 cc of a 10% solution of Brij 58 (polyoxy-dodecyl sulfate (final concentration 1%) was added. The solution fractionated by gel electrophoresis (Fig. 2). As is apparent then 0.3 ml of a 10% solution of sodium deoxycholate, pH 8.5.

Fractionation of G and N Polysomes—A modification of an earlier procedure was used (24, 25). One hundred milliliters of Chinese hamster ovary (CHO) cells in spinner culture at 4 x 10^9 cells/ml were concentrated by centrifugation (1500 x g, 5 min), washed twice in PO_4-free medium, and then resuspended in Buffer A at a concentration of 1.5 x 10^9 cells/ml. After swelling on ice for 10 min, the cells were disrupted with 40 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Nuclei were removed by centrifugation for 4 min at 4000 x g. To 1.5 ml of postnuclear supernatant was added 1.0 ml of Buffer B (0.12 M Tris, pH 8.5, 0.24 M NaCl, 0.0075 M MgOAc), then 0.3 ml of a 10% solution of sodium deoxycholate, pH 8.5. Following a 2-s mixing, 0.3 cc of a 10% solution of Brij 58 (polyoxy-dodecyl sulfate (final concentration 1%) was added. The solution fractionated by gel electrophoresis (Fig. 2). As is apparent then 0.3 ml of a 10% solution of sodium deoxycholate, pH 8.5.

Cell-free Protein Synthesis—Conditions for protein synthesis in cell-free extracts of rabbit reticulocytes (26) and wheat germ (27) have been described in detail. Conditions for studying binding of labeled VSV mRNAs to ribosomes and incorporation into polyribosomes have also been described (24).

Gel Electrophoresis of Protein—Pancreatic RNase (50 μg/ml) was added to all reactions, followed by incubation at 37° for 5 min. Generally, 5 μl of the reaction were analyzed on a 13% polyacrylamide slab gel as detailed by Laemmli (28). Samples of labeled infected cells were analyzed in a similar manner (29). The gels were fixed (30), dried, and subjected to autoradiography with Kodak Royal Blue single-side emulsion x-ray film. As described above, the autoradiograms were scanned with a Joyce-Loebel microdensitometer and the peaks quantitated by planimetry.

RESULTS

Sizes of Polyribosomes Containing G and N mRNAs in Infected Cell—In the steady state, the average number of ribosomes per messenger RNA molecule, the average polysome size, is proportional to the rate of polypeptide chain initiation and to the size of the translated region of the mRNA, and inversely proportional to the rate of chain elongation. The molecular weight of N protein (52,500) in 0.82 that of the initial unglycosylated translation product of G mRNA (Go, M_g = 63,500; Ref. 29). The two VSV mRNAs encoding these proteins can be resolved by formamide polyacrylamide gel electrophoresis (Ref. 11, 12; c.f. Fig. 2); Band II RNA (M_w = 700,000) encodes G, and Band III (M_w = 500,000) encodes N. Assuming, as is likely (c.f. Ref. 10), that the rates of elongation of nascent G and N chains are the same, then the rate of initiation of protein synthesis on each G mRNA was less than 0.82 that per N mRNA, the average size of G polysomes would be smaller than that of N polysomes.

To determine the average size of N and G polysomes, infected cells were labeled for 3 h with ^32P0_4. The postnuclear supernatant was treated with 1% sodium deoxycholate and 1% Brij to release membrane-bound polyribosomes; control experiments (not shown) indicated that this mixture of detergents had no effect on the sedimentation profile of rabbit reticulocyte polyribosomes. Following centrifugation through a sucrose gradient to resolve polyribosomes, RNA was extracted from the different regions of the gradient (Fig. 1) and quantitated by gel electrophoresis (Fig. 2). As is apparent from Fig. 2 and 1, G mRNA (Band II) is found on polysomes containing a larger number of ribosomes than is N mRNA (Band III).

Quantitation of this effect is made difficult by the fact that over 80% of the acid-precipitable radioactivity sedimenting more slowly than monoribosomes (Fractions 22 to 26, Fig. 1) is in phospholipid and tRNA, not VSV RNA. However, over
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FIG. 1. VSV-specific polysomes in infected cells. Infected cells were labeled with $^{32}$P-04, disrupted, and fractionated by centrifugation through a sucrose gradient as described under "Materials and Methods." a, profile of optical density (solid line) and acid-precipitable radioactivity (circles) per 50-$\mu l$ aliquot. Centrifugation is from right to left; monosomes are in Fraction 16, disomes in 12, trisomes in 9, and tetrasomes in 6. b, ratio of $^{32}$P radioactivity in Bands II (G mRNA) to III (N mRNA) in pooled gradient fractions. As detailed under "Materials and Methods," RNA was extracted from pooled gradient fractions and analyzed by electrophoresis through polyacrylamide gels (Fig. 2). The ratio was determined from the areas under the peaks in the scans shown in Fig. 2.

90% of the labeled material in Fractions 1 to 21 is VSV RNA (data not shown). By multiplying the total radioactivity in each fraction A to E by the fraction of radioactivity in each fraction that is in G mRNA (calculated by determining the area under the peaks in Fig. 2), one can determine the distribution of G mRNA in these polysome fractions. Calculations similar to those detailed in Table I indicate that each G mRNA which is bound to ribosomes contains, on the average, 4.76 ribosomes, each N, 4.03 ribosomes, and each Band IV RNA (M + NS), 3.3 ribosomes. Thus, the density of ribosomes per unit distance of G mRNA, relative to that of N mRNA, is $0.98 = (4.76) (52.5)/(4.03) (63.5)$. This indicates that, in the infected cell each G mRNA molecule initiates protein synthesis as frequently as does each N mRNA.

Relative Amounts of G and N mRNAs and Proteins—Villarreal et al. (15), reported that, in the VSV-infected cell, the molar ratio of G protein produced per molecule G mRNA was the same as for N protein and N mRNA. Using very different procedures for isolation of RNA from the entire cell (see "Materials and Methods") and different estimates of RNA and protein molecular weights (11, 12), we have been able to reproduce their results. Infected cells were labeled, in parallel, with $^{32}$PO$_4$ (Fig. 3) or with a mixture of $^{14}$C-amino-acids (Fig. 4). Radioactive RNA and proteins extracted from the entire cell were fractionated by polyacrylamide gel electrophoresis, and the amounts of labeled material were determined by integrating the area under the peaks from the microdensitometer scans of the radioautograms (Figs. 3 and 4). Using values we previously determined for the sizes of G and N RNA, for the N polypeptide (12) and for the polypeptide (G$_0$) which is the unglycosylated translation product of G mRNA (29), we calculate (Table I) that the molar ratio of G:N proteins made in the infected cell is about 0.68, and the molar ratio of G:N mRNAs is about 0.55. It is apparent that the yield of polypeptide per molecule of G and N mRNAs are very nearly the same. The major problems with this experiment concern the uncertainties in the molecular weights of these RNAs and proteins, and in the unproven assumption that all RNAs and proteins are extracted from the cell at the same efficiency.

Polysomes Containing G and N mRNAs in Reticulocyte Cell-free System—Crude cell-free extracts from rabbit reticulocytes translate endogenous globin mRNA and exogenous mRNAs at high efficiency (31); each mRNA is translated 10 to 50 times during a 30-min incubation period. As was shown previously, a large fraction of total VSV mRNA is incorporated into polyribosomes in such a lysate under conditions of protein synthesis (24, 32). These polysomes contained an average of four ribosomes per mRNA (c.f. Fig. 5, Table II) similar to that obtained in intact cells (Fig. 1). The rate of chain elongation of nascent globin chains, and by inference nascent viral polypeptides, is at least one-half that observed...
TABLE I

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|-----------------------------------|------|

Relative synthesis of G and N polypeptides and mRNAs

The areas under the peaks of scans of radioautograms of polyacrylamide gels similar to those of Figs. 3 and 4 were quantitated by planimetry. Two separate experiments were done; the data from Figs. 3 and 4 are part of Experiment I. In both studies, \(^{32}P\)RNA was isolated at 180, 210, 225, 240, and 270 min of infection, and the ratios of G/N mRNAs were averaged from these five samples. The absolute value of G/N mRNAs (shown ± the standard deviation) is the ratio of the areas under the peaks in the scans of the radioautograms; the molar ratio is calculated from this value assuming molecular weight for G and N mRNAs of 700,000 and 500,000, respectively (11). "G"-proteins were isolated after 8, 12, 15, 20, and 25 min of labeling, and the average absolute ratio of G/N polypeptides was averaged from these five samples. The molar ratio was calculated assuming an M, of N of 52,500 and of the primary translation product (G,.) of G of 83,500 (12, 30).

| Ratio of G/N mRNA | Experiment I | Experiment II |
|-------------------|--------------|--------------|
| Absolute          | 0.68 ± 0.05  | 0.71 ± 0.06  |
| Molar             | 0.54         | 0.66         |

| Ratio of G/N polypeptides | Absolute | Molar |
|---------------------------|----------|-------|
| Absolute                  | 0.82 ± 0.04 | 0.85 ± 0.03 |
| Molar                     | 0.97      | 0.70   |

Fig. 3. Relative amounts of G and N mRNA in infected cells. Cells were infected with VSV and labeled with \(^{32}P\)O, as detailed under "Materials and Methods." At 240 min of infection (Panel b) and 270 min (a), cells from 0.3 ml of the infection were recovered by centrifugation. RNA was isolated from the cell pellet as detailed under "Materials and Methods," and 10% was analyzed by polyacrylamide gel electrophoresis. Shown is a scan of the radioautogram. As described in Table I, RNA samples were prepared and analyzed after other times of labeling.

Fig. 4. Relative synthesis of G and N proteins in infected cells. A portion of the VSV-infected cells employed in the experiment of Fig. 3 was used; cells were in PO,-free medium but did not contain \(^{32}P\)O. At 4 h of infection, 50 μCi \(^{32}P\)-amino-acid mixture (New England Nuclear Corp.) was added to 3 ml of infected cell culture. After 5 min (Panel b) and 15 min (Panel a) and other times (see Table I) of further incubation, cells from the culture were recovered by centrifugation. These were dissolved in buffer containing sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis (see "Materials and Methods"). Shown are scans of the radioautograms of the dried gel.

Fig. 5. Reticulocyte polyribosomes containing labeled G and N mRNA. Reactions (220 μl) containing a reticulocyte cell-free extract and all reagents necessary for protein synthesis were preincubated at 30 C for 30 s. Twenty microliters of solution containing 200,000 cpm of \(^{32}P\)-labeled G mRNA (Panels a and b) or 120,000 cpm of \(^{32}P\)-labeled N mRNA (Panels c and d) were added and incubation was continued. After an additional 1.5 min (Panels a and c) or 5.0 min (b and d) of incubation, aliquots of 100 μl were added to 1.2 ml of ice-cold Buffer D (0.5 M NaCl, 0.03 M Mg(OAc), 0.02 M Hepes, pH 7.5). These were layered atop a 36-ml 15 to 30% (w/v) sucrose gradient made up in Buffer D and were centrifuged for 3.5 h in a Beckman SW 27 rotor. The gradients were pumped through a flow cell and the optical density at 260 nm was recorded continuously; from this profile, due predominantly to the endogenous polysomes containing globin mRNA, the positions of monoribosomes and polysomes of different sizes were ascertained (numbers in Panels a and c). The entire contents of each fraction (about 1.0 ml) was counted, following addition of Aquasol (N. E. Nuclear Corp.) and water, in a liquid scintillation counter. Analysis of this experiment is in Table II (Experiment I). Complete details concerning the reaction conditions and analyses of gradients has been published (24).

suggests that in these extracts the rate of polypeptide chain initiation and elongation on each mRNA is the same.

Rates of polypeptide chain initiation in these lysates can also be determined if the reactions contain 1 mg/ml of aniso-
The polysome gradients depicted in Fig. 5 are those labeled Experiment I; Experiment II was a similar study using different preparations of RNA. In calculating the data given in Column 5, the amount of radioactivity in any region of a polysome gradient, \( C_i \), was multiplied by the number of ribosomes per polysome \( i \), that is, by the number of ribosomes translating each mRNA in that peak. Hence, the total number of ribosomes translating the mRNAs is proportional to the sum of this product over all regions of the polysome gradient sedimenting with or faster than monoribosomes. Since the total radioactivity in polysomes (Column 4) is proportional to the number of mRNAs being translated, the average number of ribosomes per bound mRNA is given by Column 6.

| Experiment | mRNA | Time (s) | cpm in mono- or polysomes | Proportionate number of ribosomes bound to mRNA | Ribosomes per bound mRNA | Total cpm recovered from gradient | Fraction of mRNA bound to ribosomes |
|------------|------|---------|---------------------------|---------------------------------------------|-------------------------|----------------------------------|----------------------------------|
| I          | G    | 1.5     | 42,300                    | 103,000                                     | 2.43                    | 80,400                           | 0.55                             |
|            |      | 5.0     | 35,446                    | 142,000                                     | 4.02                    | 75,400                           | 0.50                             |
|            | N    | 1.5     | 21,500                    | 54,000                                      | 2.51                    | 41,200                           | 0.52                             |
|            |      | 5.0     | 24,900                    | 96,501                                      | 3.88                    | 49,300                           | 0.51                             |
| II         | G    | 1.5     | 8,290                     | 21,400                                      | 2.58                    | 17,400                           | 0.48                             |
|            |      | 5.0     | 7,540                     | 24,300                                      | 3.20                    | 16,000                           | 0.47                             |
|            | N    | 1.5     | 9,260                     | 20,500                                      | 2.21                    | 16,200                           | 0.61                             |
|            |      | 5.0     | 11,600                    | 32,800                                      | 2.83                    | 23,200                           | 0.50                             |

**Table II**

Incorporation of labeled VSV G and N mRNAs into polysomes

**Table III**

Rate of incorporation of G and N [32P]mRNAs into 80 S initiation complexes

Reactions and method of analysis have been described in detail (24). Reticulocyte cell-free reaction (300 μl) containing 1 mg/ml of anisomycin were incubated at 25° for 30 s. Twenty microliters of water containing about 10⁶ cpm of G or N [32P]mRNA were added. After further incubation for 15, 30, or 240 s, an aliquot of 100 μl was added to 1.2 ml of ice-cold Buffer D (see legend to Fig. 5). Gradient analysis was as in Fig. 5, except centrifugation was at 16,000 rpm for 20 h. Shown is the fraction of 32P radioactivity sedimenting under the 80 S monosome peak. In the absence of incubation, between 0.02 and 0.04 of the added labeled RNA sedimented with 80 S monosomes. This background has been subtracted from all values shown. The method for calculating the time for half-maximal binding was described in detail (24).

| Seconds of incubation | Fraction of labeled RNA in 80 S initiation complex |
|-----------------------|--------------------------------------------------|
|                       | G mRNA                                           | N mRNA                                           |
| Experiment I          |                                                 |                                                 |
| 15                    | 0.268                                            | 0.239                                            |
| 30                    | 0.394                                            | 0.422                                            |
| 240                   | 0.899                                            | 0.900                                            |
| Time for half-maximum binding (seconds) | 16.8 ± 0.6 | 16.0 ± 1.8 |
| Experiment II         |                                                 |                                                 |
| 15                    | 0.270                                            | 0.285                                            |
| 30                    | 0.424                                            | 0.456                                            |
| 240                   | 0.554                                            | 0.534                                            |
| Time for half-maximum binding (seconds) | 16.8 ± 1.8 | 16.9 ± 0.9 |

Aurintricarboxylic acid, at moderate concentrations, is a specific inhibitor of polypeptide chain elongation (24). Under these conditions, a large fraction of added VSV mRNA is incorporated into 80 S ribosome initiation complexes. These complexes remain stable in a solution of 0.5 M NaCl and 0.08 M magnesium acetate, conditions which will dissociate 40 to 60 S ribosome couples which do not contain mRNA and initiator tRNA. Table III shows that the extent of formation of 80 S complexes, and the rate at which these are formed, is the same for G and N [32P]mRNAs. This is additional evidence that these mRNAs initiate protein synthesis at the same rate in reticulocyte cell-free extracts.

Results very similar to those in Fig. 5 and Tables II and III have been obtained using the wheat germ cell-free system (data not shown).

**Effects of Inhibitors of Polypeptide Chain Initiation on Cell-free Synthesis of VSV N and G Proteins—**Recently we have derived kinetic equations for the rates of initiation, elongation, and termination of polypeptide chains in mammalian cells (1–3). This model is based on the assumption that different mRNAs may have different affinities for Met-\(\text{tRNA}^{40 S}\) complexes which can bind mRNA. Fig. 6 shows the time for half-maximal binding (seconds) of ATA, synthesis of G (and M) proteins is inhibited almost twice as much as is synthesis of N or NS proteins (Fig. 6, Table IV). (So little L protein is made that the effects of ATA on it could not be quantitated.) Comparable results were obtained in the nuclease-treated (34) reticulocyte extract (data not shown).

A large number of synthetic polyribonucleotides and polydeoxyribonucleotides, such as poly(dT), are also specific inhibitors of chain initiation, and also block binding of mRNA to the Met-\(\text{tRNA}^{40 S}\) complexes which can bind mRNA (33), while translation of all VSV mRNAs in the wheat germ cell-free system is inhibited, at higher concentrations of ATA, synthesis of G (and M) proteins is inhibited almost twice as much as is synthesis of N (or NS) proteins (Fig. 6, Table IV). (So little L protein is made that the effects of ATA on it could not be quantitated.) Comparable results were obtained in the nuclease-treated (34) reticulocyte extract (data not shown).
translation of VSV G (and M) mRNAs.

Extrapolation of the data in Fig. 6 and Table IV to complete inhibition, either by ATA or poly(dT), indicates that translation of M or G mRNAs would be inhibited 2-fold more than that of N or NS mRNA.

These results show that G mRNA is up to 2-fold less efficient than N mRNA in initiating in vitro protein synthesis under conditions where certain (essentially unknown) components of the initiation machinery are made rate-limiting.

**DISCUSSION**

The aim of the present investigations was to answer a supposedly simple question: do VSV G and N mRNAs differ in their abilities to initiate synthesis of polypeptide chains? Polypeptide chain initiation in eukaryotic cells is an extremely complex process, requiring at least eight initiation factors, mRNA, initiator Met-tRNA, 40 S and 60 S ribosome subunits, GTP, and ATP (35). It appears that, at present, one cannot assign a single number "rate constant for chain initiation" to a particular mRNA, rather, the overall rate of chain initiation can be determined only with reference to a particular constellation of concentrations of factors, ribosomes, subunits, and so forth, which exist in a particular cell or cell extract. Changes in any rate-limiting components of the initiation machinery can affect not only the overall rate of the process, but can affect the relative translatability of different mRNAs. Concentrations and activities of these components can vary between cell types, or between different physiological stages of a single cell; it may not be possible to determine or to define a "normal" set of concentrations or activities. Changes in the concentrations or activities of factors involved in chain elongation can also affect the overall rates of initiation of protein synthesis, as well as affect the relative synthesis of proteins by different mRNAs (1-3).

None of the experiments on the relative function of VSV N and G mRNAs, either in this paper or previously published by other groups, is completely free of assumptions or problems, as is discussed above. All of them are consistent with the notion that, under the conditions which occur in the infected cell or in the reticulocyte cell-free system, the rates of chain initiation (and elongation) on the two mRNAs is the same. These may be summarized as follows: (a) The molar ratio of G to N protein made in infected cells is very similar to the molar ratio of G to N mRNA recovered from these cells (Ref. 15; Figs. 3 and 4, Table I). (b) In the infected cell, the average size of polysomes synthesizing G protein is about 20% larger than that synthesizing N. Thus, the density of ribosomes on the translated part of each mRNA seems to be the same (Figs. 1 and 2). (c) The rate of binding of G and N mRNAs to reticulocyte ribosomes and formation of 80 S initiation complexes is the same (Table III). (d) The size of reticulocyte polysomes containing G and N mRNAs, and the rate at which these polysomes are formed in vitro, is the same (Fig. 5, Table II).

However, two types of experiments show that, under certain conditions of inhibition of the overall rate of chain initiation, G mRNA is translated less efficiently (about 2-fold less) than N mRNA. First, treatment of infected cells with media containing hypertonic salt results in inhibition of the rate of
chain initiation. The mechanism by which this occurs is not known (20-22). Under these conditions, synthesis of G protein is inhibited twice as much as is N protein (19). Second, addition to a wheat germ (Table IV) or reticulocyte extract (data not shown) of inhibitors, such as poly(dT) or ATA, results in inhibition of initiation of translation on all mRNAs, but translation of G mRNA is inhibited about twice as much as is that of N mRNA. It is not known precisely how these compounds inhibit polypeptide chain initiation. They do not affect the binding of the initiator Met-tRNA, to the 40 S ribosome subunit, but they do block subsequent binding of the mRNA (23). It is clear that N and G mRNAs differ in their affinities toward, or requirements for, some component required for chain initiation, possibly that substance(s) whose function is blocked by ATA, poly(dT), or hypertonic salt.

The following model is speculative, but it does serve to interrelate and explain these sets of apparently disparate observations. For simplicity, consider the case where only two mRNAs, mRNAN and mRNAG, are being translated. Binding of Factor F to these mRNAs is assumed to be a prerequisite for initiation of protein synthesis. This binding is assumed to be in equilibrium, and these two mRNAs differ in the equilibrium constant, $K_G$ or $K_N$, for binding of this factor:

\[
\text{mRNA}_G + F \rightleftharpoons K_G \text{ (mRNA}_G F) \tag{1}
\]

\[
\text{mRNA}_N + F \rightleftharpoons K_N \text{ (mRNA}_N F) \tag{2}
\]

Rates of all subsequent stages of chain initiation, binding to 40 S-Met-tRNA subunits, addition of 60 S subunits, are assumed to be the same. Thus, the relative rates of translation of the two mRNAs will be proportional to the relative concentrations of the (mRNA - F) complex. If $G$, and $N$, represent the total amount of G and N mRNAs, then the amounts of the two complexes will be given by

\[
\frac{(\text{mRNA}_G F)}{1 + \frac{1}{K_G}} = \frac{G}{1 + \frac{1}{K_G}} \tag{3}
\]

\[
\frac{(\text{mRNA}_N F)}{1 + \frac{1}{K_N}} = \frac{N}{1 + \frac{1}{K_N}} \tag{4}
\]

Assume further that $K_G < K_N$, i.e., that N mRNA has a higher affinity for F than does G mRNA. If F is much greater than $K_G^{-1}$, then the ratio of the mRNA-G-F complexes will approach $G/N$; thus, the two mRNAs will be translated at almost equal efficiencies. At very low values of F, the ratio will approach $(G_N)/(N_G)/(K_G)/(K_N)$ or the mRNAs will be translated in proportion to their constants for binding Factor F. Thus, reduction in the concentration of Factor F will inhibit, preferentially, translation of that mRNA, say G mRNA, with the lower binding constant. Similar results are obtained if $K_G$ and $K_N$ are assumed to be forward rate constants, when the subsequent binding of 40 S ribosomes to the (mRNA - F) complex follows so closely that the above reactions are not in equilibrium (3).

This model will explain satisfactorily all of the observations concerning the relative translation of G and N mRNAs if it is to be assumed that (a) $K_G = 0.25$ to 0.5 $K_N$; (b) F is in excess in the infected cell and in the cell-free system; and (c) ATA, poly(dT), and hypertonic salt all act, directly or indirectly, to block the action of F. This explanation is obviously speculative since it is not clear which, if any, of the initiation factors binds directly to mRNA, nor what the sites of action of these inhibitors might be. This model is a more specific version of one proposed recently (1, 3) and is similar to one used by Golini et al. (36) to explain the differential translation (37, 38) of picornavirus mRNA and cellular mRNA in the infected cell. They showed that cell-free translation of EMC RNA requires much less initiation factor eIF-4B than does the typical cellular mRNA. When eIF-4B is limiting, EMC RNA is translated in preference to cell mRNA. They postulate that infection of cells by EMC virus results in inactivation of Factor eIF-4B, but this has not been tested directly.

Whether or not this type of model is correct, it is clear that neither the hypertonic salt-shift treatment of cells, nor the use of inhibitors of chain initiation in vitro, will invariably yield information concerning the relative rates of chain initiation in the normal, unperturbed cell.

A final point concerns structural features of N and G mRNAs which could be related to these differences in rates of chain initiation. All VSV mRNAs have the same nucleotide sequence at the 5' end; m²G(5')pppA(m)pGpApGp, and a poly(A) sequence of about 100 bases at the 3' end (13), so these regions are unlikely to be involved in these differences in function. The AUG initiation codon in N mRNA is 13 nucleotides from the 5' end, and the m²G-containing "cap" sequence is contained within the region protected by 80 S initiation complexes from nuclease digestion. By contrast, the AUG initiation codon in G mRNA is greater than 19 nucleotides from the 5' end, and the 5' "cap" is not protected by the 80 S ribosomes (34). The presence of the m²G residue does enhance the rate of chain initiation by VSV mRNAs by mammalian ribosomes (24, 32, 39), and it is possible that the closer proximity of the 5' cap to the AUG initiator codon in N mRNA enhances its affinity for some initiation factor, possibly one which binds to, in part, the m²GppX sequence on mRNA.

In the infected cell, polysomes containing G mRNA are localized to the endoplasmic reticulum, while N mRNA is found on free polysomes (16, 17). Attachment of the G mRNA-ribosome complex to membranes is mediated by the nascent protein chain (25), and initiation of translation of G mRNA occurs with ribosome subunits which need not be attached to membranes (40). The localization of G mRNA to membranes thus seems unlikely to affect, directly, the process of polypeptide chain initiation, although initiation on G mRNA could be facilitated by a pool of 40 S ribosome subunits bound to the endoplasmic reticulum (41).

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