RIBOSOMAL FACTORS EFFECTING THE STIMULATION OF CELL-FREE PROTEIN SYNTHESIS IN THE PRESENCE OF FOOT-AND-MOUTH DISEASE VIRUS RIBONUCLEIC ACID*

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SUMMARY

Ribosomal associated factors dissociated with high salt mediate the binding of N-acetyl-aminoacyl-tRNA to salt washed (s-) ribosomes in the presence of exogenous messenger RNA (*e.g.*, foot-and-mouth disease virus (FMDV)-RNA). Only ribosomal factors isolated from FMDV-infected cells could show a stimulation of N-acetyl-aminoacyl-tRNA binding in the presence of FMDV-RNA. Amino acid incorporation studies also corroborated the need for ribosomal associated factors from FMDV-infected cells for stimulation. Polyacrylamide gel analysis of the cell-free product synthesized in the presence of FMDV-RNA indicated that amino acid labeled protein appeared to be a large molecular weight and rather heterogeneous protein.

Recently, a substance isolated from poliovirus infected HeLa cells has been shown to interfere with the initiation of cell-free synthesis of hemoglobin (1). This inhibitory substance suggests the need for (a) virus specific initiation factor(s) insensitive to any virus specific inhibitor of host cell translation. Accordingly, ribosomal factors, present in phage-infected cells, appear to be involved in the translation of virus-specific polypeptides (2,3).

Several laboratories have reported evidence for specific, ribosomal-associated factors dissociated from ribosomes by high salt. Such factors

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(termed I-factors), isolated from reticulocytes, initiate *de novo* hemoglobin synthesis (4,5). Previously, we have reported that baby hamster kidney I-factors can stimulate FMDV-RNA dependent amino acid incorporation (6). This report will present evidence suggesting the involvement and specificity of I-factor(s) that stimulate the binding of N-acetylated aminoacyl-tRNA, as well as incorporation of amino acids into polypeptides in the presence of exogenous FMDV RNA at low magnesium concentrations. Several reports have recently described a mammalian cell-free system capable of synthesizing virus specific proteins in response to exogenous encephalomyocarditis (EMC) virus RNA (7-9). These systems, unlike ours, do not require additional ribosomal factors for translation. The latter report (8), however, suggests a tissue specific factor for the initiation of EMC virus-RNA translation.

MATERIALS AND METHODS

Ribosomes from pre-chilled (8° for 120 min) uninfected baby hamster kidney cells or guinea pig livers were dissociated with 0.5 M KCl using the previously published procedure of Ascione and Arlinghaus (6). These 1 X s-ribosomes were then pre-incubated according to the method described by Ascione *et al.* (10), and designated as 2 X s-ribosomes.

I-factors were prepared from 20 to 30 mg/ml of 1 X-polyribosomes isolated as described previously (6) and dissociated (at 1°) with molar KCl containing 10 mM $MgCl_2$, 1 mM DTT, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM GTP and 30% glycerol, and concentrated as described previously (10).

A mixture of 18 labeled ^{3}H-amino acids supplemented with 2 additional ^{12}C-amino acids were esterified using a guinea pig pH 5 enzyme fraction supplemented with unfractionated yeast or guinea pig liver tRNA. Each reaction mixture (1 ml) contained: standard buffer and energy generating system (6), 0.1 mM coenzyme A, 20 A_{260} units of ^{3}H-labeled aminoacylated-tRNA (32,000 dpm/A_{260} unit), 30 A_{260} units of s-ribosomes prepared from guinea pig liver or baby hamster kidney ribosomes (10), 300 µg of FMDV-RNA,

1.5 mg of guinea pig (ribosome-free) supernatant-protamine sulfate treated enzyme (70% ammonium sulfate precipitated) (6), and 700 µg of I-factor as indicated. After incubation, samples were applied to Whatman 3 MM filter paper discs and prepared for counting as described elsewhere (11).

The complete binding reaction mixture (200 µl) contained: 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0, 40 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 60 µg of FMDV-RNA, 0.2 µmoles of GTP, 3.0 A_{260} units of a reconstituted mixture of N-acetylated 18-^{3}H-aminoacyl-tRNA (32,000 dpm/A_{260} unit), acetylated according to the procedure of Haenni and Chappeville (12), 200 µg of I-factor, and 1 to 6 A_{260} units of 2 X washed s-ribosomes as indicated. The binding reactions were incubated at 37° for 5 min and stopped by addition of 20 volumes of ice-cold binding assay buffer solution; 30 mM HEPES, pH 7.0; 40 mM KCl, 4 mM $MgCl_2$, and 0.4 mM EDTA. The diluted samples were membrane-filtered and washed using 40 more volumes of the above buffer. After drying, the filters were counted using a Triton X-100-toluene based counting fluid at 24% efficiency for ^{3}H- and 70% efficiency for ^{14}C-isotope.

Baby hamster kidney-21, clone 13 cells, obtained from the American Type Culture Collection were grown in 2 one-liter Baxter bottles as previously described by Polatnick and Bachrach (13).

Milligram quantities of FMDV-RNA were prepared from decigram quantities of purified FMDV (14) using the sodium dodecylsulfate (SDS) pH 5.0 sodium acetate buffer extraction procedure of Arlinghaus *et al.* (23).

After 30 min or more of incorporation at 37°, the labeled product was removed from ribosomes by incubating the reaction with 100 µg/ml of ribonuclease and 10 mM EDTA for 30 min (at 37°) and then centrifuged and isolated from the 10^5 x g supernatant using 10% TCA. The TCA-precipitated samples were treated according to the method of Maizel *et al.* (16) and electrophoresed on a 7.5% acrylamide gel for 17 hr at 8 ma/gel. As a marker, uniform ^{14}C-FMDV protein (type A-119) was labeled *in vivo* and isolated as described by Vande Woude and Bachrach (15).

RESULTS

Using an assay similar to the one described by Ayuso and Heredia (17) one is able to observe the stimulation by FMDV-infected cell I-factor of the N-acetylated-^{3}H-aminoacylated-tRNA binding to s-ribosomes. The binding is sensitive to low concentrations of aurintricarboxylic acid (ATA) and the I-factors were inactivated by heating at 80° for 2 min. However, the binding reaction was relatively insensitive to low concentrations of cycloheximide and pre-incubation with ribonuclease. Moreover, using N-acetylated derivatives, the binding reaction was specific for I-factors and the transferase factors could not stimulate binding. At high Mg^{++} concentrations >5 mM, the need for I-factors was diminished since the labeled N-acetylated-aminoacyl-tRNA bound non-enzymatically. This effect is reminiscent of the optimal N-formyl-methionyl-tRNA binding that takes place in the presence of ribosomal initiation factors at low magnesium concentrations (18).

Consistent with other studies (4,5) with reticulocytes, we have also noted that a stimulation in the rate of amino acid incorporation by baby hamster kidney and guinea pig liver I-factors was observable only at low magnesium concentrations. At high magnesium concentrations, I-factors were not required for incorporation.

To examine the specificity of FMDV-RNA and I-factor interaction, a binding assay similar to the one described above was performed (Table 1). This experiment indicates that I-factors from various noninfected cells did not significantly stimulate the binding of the mixture of N-acetylated-aminoacyl-tRNA. A mixture of FMDV-infected baby hamster kidney cell I-factor with a noninfected organ cell I-factor did not noticeably inhibit the FMDV-RNA dependent binding, nor did varying the source of s-ribosomes.

I-factors derived from noninfected and infected baby hamster kidney cells were tested by amino acid incorporation experiments for their ability to support translation of FMDV-RNA message. The data indicate (Fig. 1)

TABLE 1. I-FACTOR SPECIFICITY OF FMDV-RNA DEPENDENT BINDING WITH A MIXTURE OF N-ACETYLATED AMINOACYL-tRNA

Ribosomal I-factor source	dpm N-acetyl-^{3}H-aminoacyl-tRNA bound	
	- FMDV RNA	+ FMDV RNA
None	320	180
Baby hamster kidney cell (uninfected cell)	1,500	1,920
Baby hamster kidney cell (infected cell)	1,410	3,750
Guinea pig liver	1,320	750
Mouse liver	1,420	1,090
Whole calf kidney	880	970
Mixture of baby hamster kidney infected cell + mouse liver	1,710	3,720

I-factor was prepared from FMDV-infected baby hamster kidney cells after at least 5 hr of infection by the overnight dissociation procedure described in Materials and Methods. Binding is expressed as dpm N-acetyl-^{3}H-L-aminoacyl-tRNA bound per 10 A_{260} units of guinea pig liver or uninfected baby hamster kidney cell 2 X s-ribosomes (after 15 min at 37°).

that infection associated I-factor markedly stimulated the incorporation of amino acids after addition of the virus message (Fig. 1). Although a rather high level of incorporation was observed with the I-factor minus FMDV RNA, this may be due to the presence of some endogenous messenger RNA. With infected cell I-factor in the absence of s-ribosomes, or the guinea pig liver I-factor plus s-ribosomes, incorporation was unstimulated by the addition of FMDV-RNA.

Thus having established conditions which apparently favored FMDV-RNA stimulated protein synthesis, we examined the cell-free protein product by polyacrylamide gel electrophoresis (Fig. 2). ^{14}C-Protein from purified FMDV labeled *in vivo* (15) was co-electrophoresed as a marker. The major

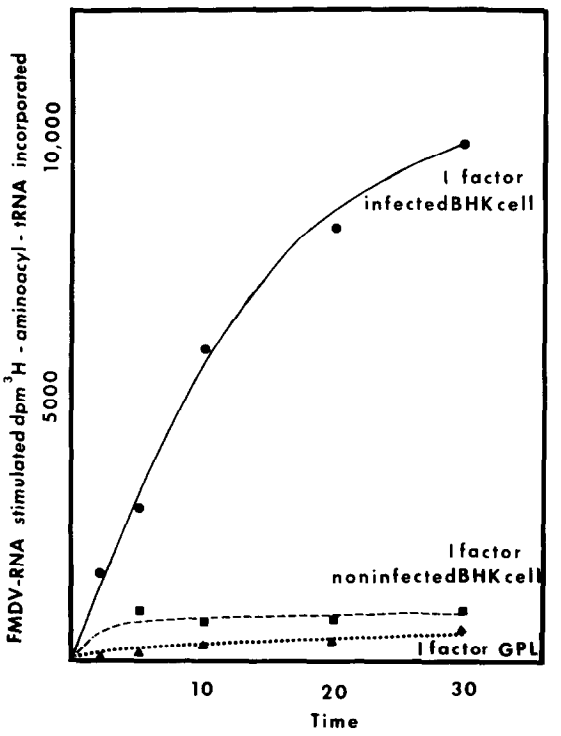

Fig. 1. FMDV-RNA dependent incorporation of a mixture of 18 ^{3}H-labeled amino acids into polypeptides in the presence of I-factors from a variety of sources. The incorporation is expressed as dpm of amino acids transfered from a mixture of 18 ^{3}H-L-aminoacyl-tRNA into a hot TCA insoluble product in the presence of FMDV-RNA (300 µg/ml) after subtracting any incorporation of amino acids in the absence of exogenous (FMDV-RNA) message.

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^{3}H-labeled product is heterogeneous and is in the 100,000 to 150,000 dalton size range; no component(s) of a similar size are present in the virus ^{14}C-protein marker. Several smaller ^{3}H-labeled products are present in the size range of the minor and major polypeptides of the ^{14}C-FMDV capsid protein (15). Although not shown, the gel profiles of the cell-free product synthesized in the absence of FMDV-RNA were completely devoid of any large molecular weight material.

DISCUSSION

The data presented in this paper indicate that a possible selection mechanism may exist for the translation of an animal virus-RNA genome and

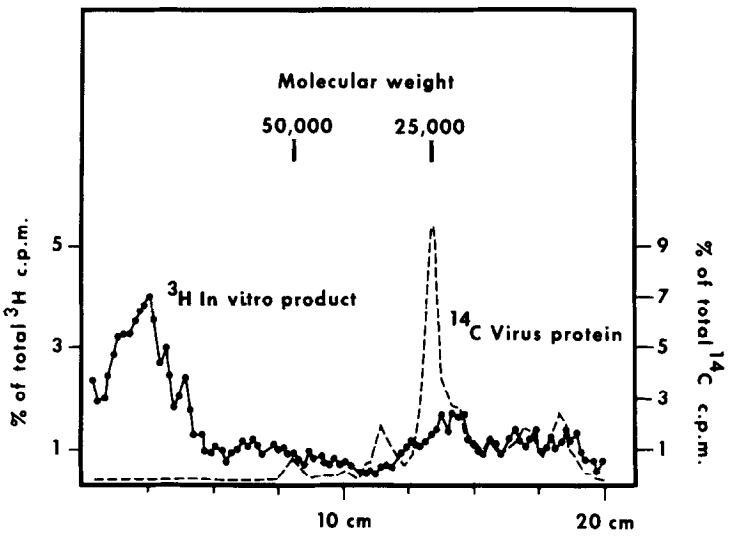

Fig. 2. Polyacrylamide gel analysis of FMDV-RNA stimulated amino acid labeled polypeptide products represented as per cent total counts ^{3}H-aminoacyl-tRNA and ^{14}C-virus protein. A scaled up (2.5 ml) incorporation reaction mixture was prepared as outlined in Materials and Methods, and product isolated and analyzed as described. Total ^{3}H-cpm in gel = 20,000; ^{14}C-cpm in gel = 4,500.

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are consistent with observations made earlier in phage-infected systems (2,3). Furthermore, such a selection mechanism appears to be mediated by ribosome factors (I-factors) similar to tissue specific factors (22) which suggest they are operative at the level of translation and possibly polypeptide chain initiation.

Binding experiments presented in this paper should not be interpreted to mean that the initiation of FMDV-RNA dependent protein synthesis requires a specific blocked α-aminoacyl-tRNA such as the N-acetylated aminoacyl-tRNA. Acetylated tRNA was used in order to preclude the possibility of binding by transferase enzymes which contaminated our I-factor preparations. Using acetylated aminoacyl-tRNA enabled us to detect the specificity involved in FMDV-RNA stimulated binding mediated by unfractionated I-factor preparations.

The predominance of the 100,000 to 150,000 dalton products prepared *in vitro* may indicate that FMD virus-specific proteins were translated into large polypeptide precursors as has been shown for poliovirus and other

picorna virus-specific proteins *in vivo* (19,20). In agreement with this, EMCV-RNA directed cell-free systems do not synthesize identifiable virus coat proteins (8,9). Thus, FMDV- and EMCV-RNA directed cell-free systems appear to be unlike the phage RNA stimulated systems which synthesize mostly (phage) coat proteins (21).

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