Initiation of Picornavirus Protein Synthesis in Ascites Cell Extracts

(translation in vitro/Met-tRNA/trypic mapping/encephalomyocarditis RNA)

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ABSTRACT The current model of picornavirus protein formation implies that initiation of protein synthesis occurs at a single site on the viral RNA, and that the large polypeptide formed is later cleaved. A direct test of this model was made in vitro by studying the incorporation of [3H]methionine from rabbit liver Met-tRNA\textsuperscript{Met} and fMet-tRNA\textsuperscript{Met} into encephalomyocarditis virus RNA-coded proteins in extracts of Ehrlich ascites cells. The incorporation of N-formylmethionine was complete within 5 min, while utilization of Met-tRNA\textsuperscript{Met} continued for 20 min. Tryptic digests of [3H]methionine-labeled products from Met-tRNA\textsuperscript{Met} analyzed by anion-exchange chromatography yielded more than 30 peptides, as compared to about 15 [3H]methionine-labeled peptides from purified encephalomyocarditis virus. In contrast, products labeled with fMet-tRNA\textsuperscript{Met} yielded one major 35S-labeled trypitc peptide. The N-terminal location of methionine in this peptide was verified by Edman degradation. One predominant N-terminal trypitc peptide was also obtained with fMet-tRNA\textsuperscript{Met} when mouse Elberfeld and mengo-virus RNAs were used as messengers. On the basis of N-terminal compared with internal labeling of the products, no evidence for in vitro post-translational cleavage was found. The results are consistent with a single initiation site for synthesis of picornavirus proteins.

One model of picornavirus protein formation involves initiation of synthesis at a single site on the viral RNA, translation of the entire genome by the initiating ribosomes, and cleavage of the resulting polypeptide to smaller functional polypeptides. This hypothesis has been supported by the isolation of large precursors to the capsid proteins (1–4) and by kinetic analyses with pactamycin, an inhibitor of initiation of protein synthesis (5–7). Translation of viral RNAs in ascites cell extracts provides a system in which the number of initiation sites on picornavirus RNA molecules can be tested directly. By a method analogous to that used by Lodish (8) to determine the number of initiation sites on bacteriophage f2 RNA in Escherichia coli extracts, f-[35S]Met-tRNA\textsuperscript{Met} has been used to label specifically the N-termini of proteins synthesized in ascites cell extracts in response to picornavirus RNA as messenger. Tryptic digests of the labeled proteins have been analyzed, and the number of N-terminal peptides has been determined. This system may also be useful for studying initiation of eukaryotic protein synthesis with other mRNAs.

MATERIALS AND METHODS

\textsuperscript{3}H-Methionine (18–31 Ci/mmol) was purchased from Amersham–Searle. [\textsuperscript{3}H]Amino acid mixture minus methionine was from New England Nuclear Corp. (NET-250). Stripped rabbit liver tRNA was from General Biochemicals. Creatine phosphate, ATP, and GTP were obtained as the Tris, potassium, and lithium salts, respectively, since Na\textsuperscript{+}– at a concentration of only 2 mM—inhibited in vitro protein synthesis almost completely. Benzoylated DEAE-cellulose was from Schwarz–Mann.

[35S]Met-tRNA. Rabbit-liver tRNA was charged with \([\text{35S}]\)methionine with calf-brain enzyme, and Met-tRNA\textsuperscript{Met} and Met-tRNA\textsuperscript{Met} were separated on benzoylated DEAE-cellulose (9). \([\text{35S}]\)Met-tRNA\textsuperscript{Met} was formlated with Ca-Lucovin (Lederle) as formyl donor and E. coli trans-formylase (10). \([\text{35S}]\)Met-tRNA\textsuperscript{Met} was also prepared for subsequent formylation by charging rabbit-liver tRNA with [35S]methionine and E. coli charging enzyme (11), which does not charge rabbit tRNA\textsuperscript{Met} (11), then purifying the Met-tRNA\textsuperscript{Met} on benzoylated DEAE-cellulose. The contamination of Met-tRNA\textsuperscript{Met} in Met-tRNA\textsuperscript{Met} was less than 5% and in fMet-tRNA\textsuperscript{Met} less than 3%, as determined by the extent of formylation (12). All of the preparations of tRNA were precipitated with ethanol and dissolved in sterile water. tRNA\textsuperscript{Met} was also charged and formylated in one step with the E. coli enzyme and formyl donor. To reduce the amount of nonformylated methionine-tRNA to less than 3%, some preparations of f-[35S]Met-tRNA\textsuperscript{Met} were incubated with 2 mM CuSO\textsubscript{4} at pH 5.0 for 15 min at 37° and precipitated with ethanol (13). The same results were obtained with tRNA prepared by the different methods.

Viral RNA and Cell-free Extracts. The preparation of viral RNAs has been described (14). An incubated S10 extract was prepared from Ehrlich ascites cells and used for in vitro protein synthesis (14).

Edman Degradation. After tryptic digestion of the in vitro products (14), formyl groups were removed by incubation in 1 M HCl in 15% methanol for 2 hr at 37° (15), then lyophilized before modified Edman degradation (16). The phenylthiohydantoin–amino acid was analyzed by extraction with ethyl acetate or by paper chromatography (16), with identical results.

Anion-Exchange Chromatography. Tryptic digests of the in vitro products were lyophilized and dissolved in 1 ml of starting buffer a, which includes per liter: 10 ml of pyridine, 15 ml of N-ethylmorpholine, 20 ml of \(\alpha\)-picoline, and acetic acid to pH 9.4. After the sample was adjusted to pH 10, it was applied to a 1 × 60-cm column of AG1-X2 (BioRad), 200–400 mesh resin. Elution at 35° (flow rate = 30 ml/hr) was performed with 75 ml of buffer a, followed consecutively by linear gradients of the following composition: \(b = 150\) ml of buffer a plus 150 ml of buffer a adjusted to pH 6.4 with acetic acid; \(c = 150\) ml of buffer a at pH 6.4 plus 150 ml of 1 M pyridine–acetic acid, pH 5.1; \(d = 150\) ml of 1 M pyridine–acetic acid, pH 5.1, plus 150 ml of 2 M pyridine–acetic acid, pH 5.1, and \(e = 150\) ml of 2 M pyridine–acetic acid, pH 5.1, plus 150 ml
clevage products of cytochrome c. After electrophoresis for 7.75 hr at 30 V, 1-mm slices were cut, dissolved in 0.1 ml of H2O2 (50%) at 60°, and mixed with 1 ml of Protosol (New England Nuclear Corp.) and 10 ml of toluene-based scintillation fluid for counting.

**RESULTS AND DISCUSSION**

Rate of methionine incorporation from Met-tRNA

The rate of encephalomyocarditis virus-specific protein synthesis was followed by measurement of the utilization of f-[35S]Met-tRNA_{Met} as compared with [35S]Met-tRNA_{Met}, by ascites cell extracts. A 1000-fold excess of unlabeled methionine was included in the incubation mixture to prevent incorporation of [35S]methionine via deacylation and recharging. As shown in Fig. 1, incorporation of methionine from fMet-tRNA_{Met} is complete within 5 min, whereas that from Met-tRNA_{Met} continues for 20 min. This finding is in agreement with previous inhibitor studies that indicated that initiation of protein synthesis in the ascites cell-free extract occurs only at the beginning of incubation, while chain elongation continues for 30 min or longer (14, 18). The basis for the failure to obtain continued formylmethionine incorporation is not known. Presumably, it is due to alterations in the viral mRNA rather than to lability of initiation factors or fMet-tRNA_{Met}, since incubation of the extracts for 1 hr instead of 30 min did not affect subsequent utilization of fMet-tRNA_{Met}, and because addition of Met-tRNA_{Met} to the reaction mixture after 5 min of translation did not result in incorporation of formylmethionine.

**Absence of post-translational cleavage in vitro**

Functional viral proteins are formed from large precursors in picornavirus-infected cells (1–4). In an attempt to determine whether a similar process occurs in vivo, products synthesized from f-[35S]Met-tRNA_{Met} and [3H]amino acids (minus methionine) were analyzed by gel electrophoresis (14). There was no significant incorporation of [35S]methionine from fMet-tRNA_{Met} in the absence of encephalomyocarditis viral RNA. As shown in Fig. 2, polypeptides of molecular weight about 100,000 (14) retained [35S], suggesting that the larger products are not cleaved. In addition, the ratio of N-terminal to internal label increased in polypeptides of smaller

**Table 1. Edman degradation of [35S]Met-tRNA-labeled products coded by encephalomyocarditis virus RNA.**

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Terminal</th>
<th>Internal</th>
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<tr>
<td></td>
<td>cpm</td>
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<tr>
<td>[35S]Met-tRNA_{Met}</td>
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<td>8.9</td>
</tr>
<tr>
<td>[35S]Met-tRNA_{Met}</td>
<td>588</td>
<td>15.7</td>
</tr>
<tr>
<td>f-[35S]Met-tRNA_{Met}</td>
<td>1310</td>
<td>80.7</td>
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0.25-ml Reaction mixtures contained 0.125 ml of ascites S10, and 15 μg of virus RNA plus 3.2 × 10^6 cpm of [35S]Met-tRNA_{Met}, 2.4 × 10^6 cpm of [35S]Met-tRNA_{Met}, and 1.0 × 10^6 cpm of f-[35S]Met-tRNA_{Met}. After incubation for 20 min at 37°, 1 ml of 0.5 M NH4OH was added and the incubation was continued for 15 min. The proteins were precipitated with CHCl3COOH, oxidized, and treated with trypsin.

The digests were hydrolyzed for 2 hr at 37° in 1 M HCl in 15% methanol, and lyophilized. Edman degradation was performed, the product was dissolved in 1 ml of H2O, and the distribution of [35S] between the water phase and 1 ml of ethyl acetate was determined. [35S] in the ethyl acetate phase is designated as terminal, and in the water phase as internal methionine.
gested with COOH, ethanol, of 20% CHCl₃COOH.

15 ml of Met-tRNAMet were synthesized [³⁵S]methionine-labeled (A).

The mode of incorporation with tRNAMet and tRNAF Met

The position of methionine in the in vitro synthesized protein with encephalomyocarditis virus RNA as template was determined by a modified Edman degradation (16). As is shown in Table 1, f-[³⁵S]Met-tRNAF Met donates methionine to the N-terminal, and [³⁵S]Met-tRNAF Met to the internal positions. Nonformylated [³⁵S]Met-tRNAF Met apparently can donate Met into internal positions under these conditions. Although the amount of Met-tRNAF Met contaminating the Met-tRNAF Met was less than 5%, as judged from the extent of formylation, incorporation of methionine from [³⁵S]Met-tRNAF Met was 7–14% of that from [³⁵S]Met-tRNAF Met. The incorporation of methionine from Met-tRNAF Met during chain elongation has been reported in rabbit reticulocyte lysates programmed with poly(A-U-G) as messenger (20), and in a noninitiating extract from mouse cells (12).

Formylated and nonformylated Met-tRNAF Met initiate protein synthesis in the same way in reticulocytes (21), but formylated methionine is not cleaved from the products. Therefore, f-[³⁵S]Met-tRNAF Met was chosen to label specifically the N-termini of encephalomyocarditis RNA-coded proteins. When f-[³⁵S]Met-tRNAF Met was incubated for 15 min at 37° C with ascites cell extract, 90% of the methionine remained formylated, indicating that the extract contained little deformylase activity.

Analysis of tryptic digests of encephalomyocarditis virus coat proteins and in vitro products

A tryptic digest of purified virus, labeled with [³⁵S]methionine was analyzed by ion-exchange chromatography (Fig. 3A). About 15–20 different peptides can be resolved, in agreement with the number observed (22, 23) when two-dimensional electrophoresis and chromatography were used. The protein synthesized in vitro with encephalomyocarditis RNA as messenger and labeled with [³⁵S]Met-tRNAF Met was similarly

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Terminal cpm</th>
<th>Terminal %</th>
<th>Internal cpm</th>
<th>Internal %</th>
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<tr>
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<td>220–230</td>
<td>2480</td>
<td>92</td>
<td>211</td>
<td>8</td>
</tr>
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</table>

Samples from the profile shown in Fig. 4 were lyophilized and subjected to Edman degradation. The amount of terminal methionine was determined by paper chromatography of the Edman degradation products.
methionine internally
35S-labeled
peak appearing
peptides
from
eluates from
Met-tRNA_FMet. (A) The total volume of the reaction mixture was 2 ml, and it contained 1 ml of ascites S10 and 1.5 × 10^7 cpm of 35S-Met-tRNA_FMet. As for A, but with 120 µg of viral RNA. Both mixtures were incubated for 10 min at 37°, then processed as in Fig. 3. 1 ml of each 3.0-ml fraction was counted.

When f-[35S]Met-tRNA_FMet was used as label for viral RNA-directed protein synthesis, the tryptic digest revealed only one major peak on the anion-exchange column (Fig. 4B). In Fig. 4A is shown a digest of proteins synthesized without added RNA. Edman degradation of the different fractions from Fig. 4B demonstrated that the incorporation of methionine is N-terminal (Table 3). The main peak in Fig. 4B, which elutes after the change to buffer d, is at the same position as the predominant peak in Fig. 3B; both are

FIG. 4. Analysis of tryptic digest of encephalomyocarditis proteins labeled with f-[35S]Met-tRNA_FMet synthesized in vitro. (A) The major peak appearing in the 35S-labeled digest of coat protein. The same pattern (but 10-fold lower radioactivity) was obtained when [35S]-Met-tRNA_FMet was used as the labeled precursor (not shown). Results of Edman degradations of lyophilized fractions from the profile in Fig. 3B and the corresponding eluates from [35S]Met-tRNA_FMet are shown in Table 2. In both cases, the peptides contain internal [35S]-methionine. Presumably, as noted above, Met-tRNA_FMet can donate methionine internally under these conditions, although the efficient utilization of a small amount of contaminating Met-tRNA_M^Met (less than 5%) could also account for the results.

FIG. 5. Gel electrophoresis of tryptic peptides translated from encephalomyocarditis virus RNA and labeled with f-[35S]Met-tRNA_FMet. (A) Samples of fractions 180–190 from Fig. 4B were lyophilized, dissolved in 0.1% sodium dodecyl sulfate–8 M urea buffer, and heated at 60° for 10 min (17). The sample was analyzed on 10-cm, 12.5% polyacrylamide gels containing 8 M urea (17). (B) Samples of fractions 210–230 from Fig. 4B were analyzed as in A. The marker peptides were CNBr-cleaved cytochrome c: a = Cyt. c I, molecular weight (MW) 7760; b = Cyt. c II, MW 2780; and c = Cyt. c III, MW 1810. Bromphenol blue is at d. Migration is from left to right.

FIG. 6. Chromatography of tryptic digest of mengo and mouse Elberfeld virus proteins labeled in vitro with f-[35S]Met-tRNA_FMet. The 1.6-ml reaction mixture contained 1 ml of ascites S10, 140 µg of mengovirus RNA (A) or 20 µg of mouse Elberfeld virus RNA (B), and 1.5 × 10^6 cpm of f-[35S]Met-tRNA_FMet. Incubation was at 37° for 10 min, and the products were processed as in Fig. 3.
absent from the ²⁸S-labeled digest of coat protein (Fig. 3A). Since the coat polypeptides are coded for by sequences at or near the 5'-terminal position of the RNA (5), these results indicate that there is a lead-in sequence of nucleotides before translation of the codons that specify coat proteins.

In order to determine their size, the peptides in peak fractions 180-190 and 210-230 in Fig. 4B were lyophilized and analyzed by gel electrophoresis on 12.5% acrylamide in 8 M urea (17). As seen in Fig. 5A and B, the peaks apparently are homogeneous, and the peptides have molecular weights of about 2000 for the main peak and 1400 for the minor peak. In view of its lower molecular weight and smaller quantity, the minor peptide may have been formed by digestion of the larger peptide.

**Initiation with mengo and mouse Elberfeld virus RNA**

Mengo and mouse Elberfeld virus RNA were also used as messengers in the ascites cell extract with [³⁵S]Met-tRNA₅⁸Met. Analysis of tryptic digests of the in vitro synthesized proteins by anion-exchange chromatography is shown in Fig. 6A for mengovirus RNA and in Fig. 6B for mouse Elberfeld virus RNA. The results correspond to those obtained with encephalomyocarditis virus RNA. In each case, only one major peak is evident, and it accounts for more than 90% of the radioactivity. The elution positions of these peptides are different from each other and from that obtained with the N-terminal tryptic peptide directed by encephalomyocarditis virus RNA. Acrylamide gel electrophoresis of the major N-terminal peptide directed by mengovirus RNA gave a homogeneous peak, with an estimated molecular weight of 2800. Thus, there appears to be no conserved initiating sequence among these three closely related viruses.

Although the unlikely possibility exists that there are several initiation points giving rise to identical N-terminal tryptic peptides, these results, in agreement with those of others—obtained by different methods (5, 24)—indicate that picornavirus RNAs have a single initiation site. It is, furthermore, probable that this initiation site is located at some distance before the sequences coding for the capsid proteins.

**NOTE ADDED IN PROOF**

A single initiation site in encephalomyocarditis virus RNA and premature termination of in vitro products has also been found by A. Smith, and by I. Boime and P. Leder (personal communication).

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