# TRANSLATION OF AN RNA VIRAL MESSAGE IN VITRO: ONE-STEP POLYPEPTIDE CHAIN ELONGATION

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Received December 16, 1970

Summary: MS2 RNA directed polypeptide chain propagation is achieved in one step as follows: The initiation reaction is first carried out under conditions that permit no peptide synthesis. Upon dilution, followed by introduction of the components required for polypeptide chain elongation, the ribosome bound to MS2 RNA initiation site proceeds to translate the cistron just once in a well-synchronized fashion (one-step elongation). It is estimated that the time required for translation of the coat cistron is approximately 12 minutes at 31°C under the conditions described; the rate of translation appears to be non-uniform. Using the same technique, aurintricarboxylic acid is shown to affect the process of polypeptide chain elongation.

INTRODUCTION: Translation of RNA viral messages in vitro occurs in three steps: initiation, elongation and termination. The components required for initiation of translation differ from those for polypeptide chain propagation. Thus, whereas ribosomes, fMet-$tRNA_f$, GTP, and $Mg^{++}$ are required in the formation of the initiation complex, additional components, such as a high speed supernatant fraction of E. coli extract, ATP, all twenty amino acids and their cognate tRNAs are needed for the latter process.[1,2]

In principle, therefore, if the initiation reaction is carried out under conditions that do not satisfy elongation, and the reaction mixture is diluted sufficiently to prevent further interaction between template RNA, initiator tRNA and ribosomes, only the formylmethionine present in the initiation complex formed prior to dilution should be converted into polypeptide chains upon introduction of the components required for elongation. Such an experiment using MS2 RNA as messenger RNA is reported in this communication, and is referred to as one-step elongation.

MATERIALS AND METHODS: Preparation of ribosomes and S165 supernatant fraction - Ribosomes and S165 were prepared as already described.[3] Unwashed ribosomes were used throughout.

Preparation of charged tRNA - fMet-$tRNA_f$ was prepared by charging E. coli $tRNA_f$ (97% pure: a gift of Dr. D. Novelli) with unlabeled or $^3H$-labeled methionine (3.3C/mmole) in the presence of leucovorin by the methods of Marcker and Sanger.[4] $^3H$-aminoacyl-tRNA was prepared similarly by charging E. coli tRNA, in the absence of leucovorin, with a mixture of twenty amino acids containing the $^3H$-labeled amino acids desired. Specific activity of $^3H$-amino acids was 3C/mmole each and radioactivities of the isolated tRNA were, per µg RNA, $1.0 \times 10^4$ CPM for tRNA mixedly charged with $^3H$-labeled alanine, glycine, isoleucine and tyrosine, $3.2 \times 10^3$ CPM for $^3H$-leucyl-tRNA, and $4.1 \times 10^3$ CPM for $^3H$-valyl-tRNA.

One-step experiment - The initiation complex was allowed to form under the following conditions: the binding reaction mixture (0.065 ml) contained 40 mM Tris (pH 7.5), 80 mM $NH_4Cl$, 5 mM Mg acetate, 4 mM β-mercaptoethanol, 2 mM GTP, 17 µµmoles of fMet-$tRNA_f$ (labeled or unlabeled as indicated in each experiment), 15 µg of MS2 RNA, and 200 µg of ribosomes (estimated from A260). Incubation was carried out for 15 minutes at 37°C. The initiation reaction was then interrupted by tenfold dilution with a medium containing Tris (pH 7.5), $NH_4Cl$, βMe, GTP, and Mg acetate (dilution medium) at the same concentration as in the binding mixture, and was equilibrated to 31°C. Polypeptide chain elongation was initiated by introduction of an equal volume of elongation mixture (0.65 ml) which contained Tris, $NH_4Cl$, GTP (each at the same concentration as in the binding mixture), 15 mM Mg acetate, 4 mM ATP, 4 mM PEP ($Na^+$ salt), 5 µg of PEP kinase, 750 µg of S165 (estimated according to Lowry[5]) and $^3H$-aminoacyl-tRNA; or alternatively, the mixture of E. coli B tRNA (250 µg) and twenty amino acids with or without $^3H$-labeled amino acid(s) as indicated in each experiment. The temperature of incubation for elongation was 31°C.

Assay for the binding and $^3H$-amino acid incorporation - The binding of $^3H$-fMet-$tRNA_f$ was determined by trapping the radioactivity on Millipore filters (HA) as described by Nirenberg and Leder.[6] Incorporation of $^3H$-amino acids was followed by measuring the hot trichloroacetic acid-insoluble radioactivity as already described.[3]

$^3H$-amino acids were purchased from Schwarz BioResearch, Orangeburg, New York, and aurintricarboxylic acid (ATA) from Aldrich Chemical Co., Milwaukee, Wisconsin.

RESULTS: The formation of initiation complex (MS2 RNA-ribosome-($^3H$)fMet-$tRNA_f$) is illustrated in Fig. 1A: maximum binding was achieved in 10 minutes at 37°C. About half of $^3H$-fMet-$tRNA_f$ was converted into initiation complex. Over 99% of the bound radioactivity was rendered acid-insoluble upon heating, indicating absence of polypeptide synthesis.

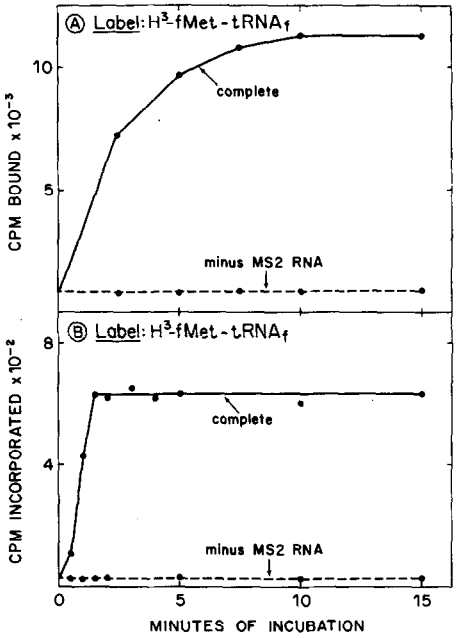


Figure 1A: Binding of $^{3}$H-fMet-tRNA$_f$ on ribosomes in the presence of MS2 RNA. Conditions as in Methods. $^{3}$H-fMet-tRNA$_f$ served as label (specific activity, 2,200 CPM per µµmole). At the time indicated, a 0.04 ml sample was withdrawn and the radioactivity bound to ribosomes was determined by trapping it on Millipore filters.

1B: One-step elongation measured by incorporation of $^{3}$H-fMet. The prebinding reaction of $^{3}$H-fMet-tRNA$_f$ was carried out for 15 minutes at 37°C as in A. Dilution of the binding mixture and initiation of elongation were carried out at 31°C as described in Methods. The elongation mixture contained E. coli tRNA and 20 unlabeled amino acids. At the time indicated, a 0.10 ml sample was assayed for hot acid-insoluble radioactivity as described in Methods.

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Figure 1B shows that upon the introduction of chain elongation components to reaction mixtures where initiation complex formation has been completed and diluted tenfold, $^{3}$H-formyl-methionine in the initiation complex was rapidly converted into hot acid-insoluble polypeptide. Conversion was complete within 1.5 minutes, indicating an excellent synchrony of the process. The absence of any further increase in hot acid-insoluble radioactivity thereafter up to 15 minutes indicates that no significant reinitiation occurred and thus illustrates one-step elongation.

The one-step experiments were performed with various $^{3}$H-aminoacyl-tRNA and are illustrated in Fig. 2A. $^{3}$H-amino acid incorporation terminates within 12 minutes after addition of the elongation mixture containing tRNA isolated after being charged with twenty amino acids, of which alanine, glycine, isoleucine and tyrosine were labeled. When $^{3}$H-leucyl-tRNA served as

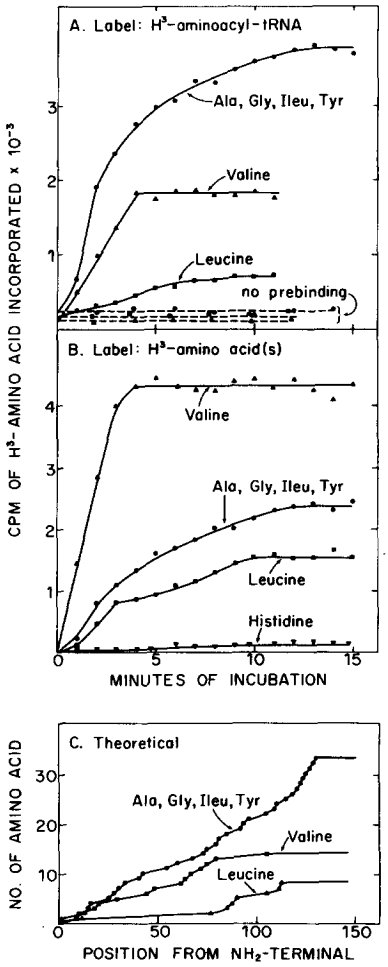


Figure 2: Experimental and theoretical one-step elongation.

2A: The experimental plan is similar to that described in Fig. 1B except that in the prebinding reaction a comparable amount of unlabeled fMet-tRNA$_f$ was substituted for the label and that the elongation mixture (0.65 ml) contained 600 µg of E. coli B tRNA precharged with 20 amino acids including the indicated $^3$H-amino acid(s). Dotted lines represent corresponding control experiments in which the prebinding reaction was omitted. Samples of 0.10 ml were analyzed for hot acid-insoluble radioactivity.

2B: Experimental conditions were as above except that 20 amino acids containing $^3$H-amino acid(s) as indicated and E. coli B tRNA were substituted for precharged tRNA in the elongation mixture, which was preincubated for 10 minutes at 31°C prior to introduction into binding mixture. Concentrations of labeled and unlabeled amino acids in the elongation mixture were 8 x $10^{-3}$ mM and 1.6 x $10^{-2}$ mM each, respectively. Specific activity of $^3$H-amino acids was 3 C/mmole when singly present and 1 C/mmole when combined. The size of samples was 0.08 ml. Incorporations when the prebinding was omitted were around 110 CPM for $^3$H-valine, 350 CPM for $^3$H-leucine, 105 CPM for $^3$H-histidine and 515 CPM for the mixture of four $^3$H-amino acids and were subtracted in the values shown.

2C: Theoretical incorporation curves for various amino acids expected from uniform rate of translation of the coat cistron were constructed by plotting cumulative number of amino acid residues against position from the $NH_2$-terminal of the coat protein according to its amino acid sequence.[18]

the label, incorporation stopped at around 9 minutes; when $^{3}$H-valyl-tRNA was used as the label, incorporation stopped as early as 4 minutes. Control experiments in which the prebinding reaction was omitted, indicated no significant incorporation of $^{3}$H-amino acids, thus reinforcing the previous conclusion that no significant reinitiation occurred under the conditions of one-step elongation experiments.

Similar results were obtained when free $^{3}$H-amino acids were used as the label (Fig. 2B). Thus, with a combination of four $^{3}$H-amino acids, termination of their incorporation again occurred at 12 minutes; incorporation of $^{3}$H-leucine and $^{3}$H-valine stopped at around 9 and 4 minutes, respectively. Incorporation of $^{3}$H-histidine was very small.

The effect of ATA on polypeptide chain growth was examined by the one-step technique. Figure 3 shows the effect of ATA on the $^{3}$H-valine incorporation at two concentrations. It can be seen that ATA effectively depressed the rate of polypeptide chain growth. With prolonged incubation, the extent of ATA inhibition was reduced; thus, ATA at a concentration of 50 μM inhibited $^{3}$H-valine incorporation by 90% at the end of 5 minutes, whereas the degree of inhibition was reduced to 31% when incubation was prolonged to 30 minutes.

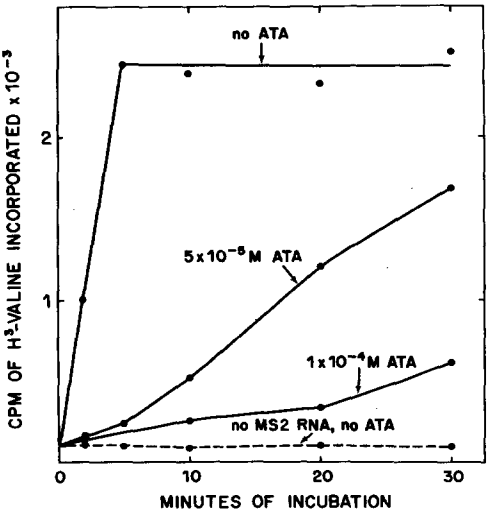


Figure 3: Effect of ATA on polypeptide chain propagation. Reaction conditions as described in Fig. 2B in which $^{3}$H-valine served as the label. ATA, when present, was added to the elongation mixture before it was introduced into the diluted prebinding mixture. At the times indicated, a sample of 0.05 ml was assayed for hot acid-insoluble radioactivity.

DISCUSSION: The data presented in this report indicate that polypeptide chain elongation can be accomplished in one step without the use of any ex-

ternal inhibitor, e.g., ATA. This is done by: (a) prebinding, (b) dilution, (c) elongation. The overall dilution is twentyfold. Under these conditions, no significant synthesis of hot acid-insoluble polypeptide is detectable without prebinding (Fig. 2A); therefore, the polypeptide chains that are synthesized originate solely from the initiation complex present prior to the dilution, and the elongation components. Hence, the time required for the incorporation of a labeled amino acid into a polypeptide to terminate in the one-step experiments represents the time required for a ribosome to travel from the initiation site to the last codon of the longest cistron coding for the amino acid employed as the label.

RNA phages carry three identified cistrons--coat protein, A protein and replicase cistrons.[7-10] The coat protein of RNA phages contains no histidine and its incorporation, when an RNA viral message serves as messenger RNA in a cell-free protein-synthesizing system, faithfully reflects the synthesis of non-coat proteins.[11,12] Since no appreciable incorporation of $^{3}$H-histidine occurs under the conditions of one-step translation (Fig. 2B), the amount of non-coat proteins synthesized must be very small. Therefore, when a combination of four amino acids (alanine, glycine, isoleucine, and tyrosine) which are present abundantly in the COOH-terminal region of the coat protein[13] is employed as the label, the time required for their incorporation to reach plateau should be the time required for a ribosome to translate the coat cistron, assuming that complete protein molecules are synthesized. Since MS2 coat protein consists of 129 amino acid residues[13,14] and the time required for the synthesis of this protein is 12 minutes (Fig. 2A,2B), the rate of translation is approximately 0.18 amino acid per second at 31°C. On the other hand, when $^{3}$H-valine is used as the label, its incorporation stops at around 4 minutes (Fig. 2A,2B). Inspection of the theoretical incorporation curve for valine illustrated in Fig. 3C, suggests that incorporation may be expected to continue until the last valine residue at the 105th position is reached; since, however, most valine residues (over 90%) appears in the region preceding the valine residue at the 79th position, it is likely that termination of its incorporation signals chain propagation up to this point. If this is so, the rate of translation of this portion of the coat cistron is 0.33 amino acid per second, which is much larger than the value obtained from the use of four amino acids. This difference may indicate a decrease in the rate of translation as ribosomes move towards the COOH-terminal, possibly as a result of the change in topography of the coat cistron. When $^{3}$H-leucine serves as label, its incorporation stops after 9-10 minutes of incubation. Since the last leucine residue is located at the 112th position,[13] a rate of 0.20 amino acid per second results; this is

in close agreement with the value obtained from the use of four labeled amino acids. The shape of the curve of incorporation, compared with that of theoretical incorporation expected from an uniform rate of translation (Fig. 2C), seems to indicate again that translation of the early part of the coat cistron is much faster than of the later part. The average rate of translation, 0.18 amino acid per second, is considerably smaller than that obtained by Webster and Zinder,[15] who assigned the value of 0.4 to 0.5 amino acid per second at 32° or 33°C with a different technique. The reason for this discrepancy is not immediately clear.

Although not shown here, twentyfold dilution still permits the binding reaction to proceed to a certain extent when monitored by the Millipore trapping of the initiation complex; however, the binding is not detected as synthesis of acid-insoluble polypeptide chains. Fortyfold dilution is required for complete elimination of the binding reaction. One-step experiments employing this dilution yield essentially the same results.

It has been shown that ATA inhibits the binding of RNA viral messages to ribosomes.[16] By the one-step experiments, ATA is demonstrated to depress the process of polypeptide chain growth, the rate in particular (Fig. 3). This effect is in agreement with reports that ATA can affect protein synthesis at steps other than messenger binding to ribosomes.[17,18] The effect is not due to the inhibition of aminoacylation of tRNA, however, since it is precharged in this experiment. The use of ATA as a specific inhibitor of initiation merits caution.

ACKNOWLEDGMENTS: The author is grateful to Drs. S. B. Weiss, R. Haselkorn and T. Nakamoto for their critical readings of the original manuscript. The excellent technical assistance of Mrs. Karen Constan is greatly appreciated.

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