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Processing of the Encephalomyocarditis Virus Capsid Precursor Protein Studied in Rabbit Reticulocyte Lysates Incubated with *N*-Formyl-[^{35}S]Methionine-tRNA$_f^{Met}$

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Translation of encephalomyocarditis virus RNA in rabbit reticulocyte lysates in the presence of *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$ revealed that a small polypeptide is cleaved from the N-terminus of the capsid protein precursor, preA, by virus-coded protease activity. Therefore, this N-terminal segment comprising the translation initiation site is not conserved in any of the mature capsid proteins.

The translation of encephalomyocarditis (EMC) virus RNA in rabbit reticulocyte lysates and the processing of the translation products conforms quite closely to the pattern in infected cells, but one major exception is that the first capsid precursor protein (which has been termed preA [5] or A1 [10]) produced in vitro is somewhat larger than the capsid precursor, A, normally seen in infected cells (5, 10). The same result was observed when translation was in extracts of uninfected Krebs ascites cells (4, 12), but in the case of extracts from infected cells, only A and not preA was detected (4, 12). In some experiments, a small polypeptide of molecular weight 12,500 could also be detected (4). The relationship of preA to A has been controversial, and there is some doubt as to whether preA is an artifact of in vitro translation. Lawrence and Thach suggested that A was derived from preA by the removal of the 12,500-dalton polypeptide from the N-terminal region of preA (4). On the other hand, when translation was carried out in the presence of *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$, preA, A, and B all appeared to be labeled (5), which suggests that the N-terminus of preA is conserved during the first steps of processing (Fig. 1a), and this is the view which seems to have gained general acceptance (2, 7–10). However, on reexamining this question, we find that only preA is efficiently labeled under these conditions. We show here that the conversion of preA to A involves the cleavage of a small fragment from the N-terminal end (Fig. 1b), which we will call L (for leader polypeptide) to avoid prejudging whether it may be identical to any of the small products previously identified in translation experiments (5, 10, 12).

EMC RNA (a generous gift from R. A. Laskey, MRC Laboratory of Molecular Biology, Cambridge, U.K.) was translated at 30°C in a nuclease-treated rabbit reticulocyte lysate prepared as described previously (6) and supplemented with 60 μg of calf liver tRNA per ml and 2 mM dithiothreitol. *N*-Formyl-[^{35}S]methionine-tRNA$_f^{Met}$ was prepared by incubation of reticulocyte tRNA with partially purified *Escherichia coli* activating enzymes and [^{35}S]methionine (1,200 Ci/mmol; Amersham International) as described by Stanley (11). Analysis of the T_1 RNase digestion products by paper electrophoresis (11) showed that the labeled material was about 97% pure, with 3% nonformylated [^{35}S]methionine-tRNA$_f^{Met}$ contaminant. It was added to translation assays at a final concentration of 8×10^7 cpm/ml. Samples of the translation assays were analyzed by gel electrophoresis as described previously (5, 6), and the gels were fluorographed by the method of Laskey and Mills (3).

PreA was the only predominant labeled product during the first 40 min of translation in the presence of *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$; on further incubation, this disappeared, and a small labeled polypeptide, L, appeared (Fig. 2). Controls in which [^{35}S]methionine was also

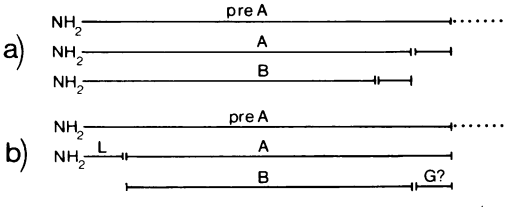

FIG. 1. Cleavage map showing the early steps in processing of EMC capsid precursor polypeptide (a) as proposed in previous work (5, 7–10) and (b) as proposed in this paper. NH_2 denotes only the N-terminus originating from initiation of translation.

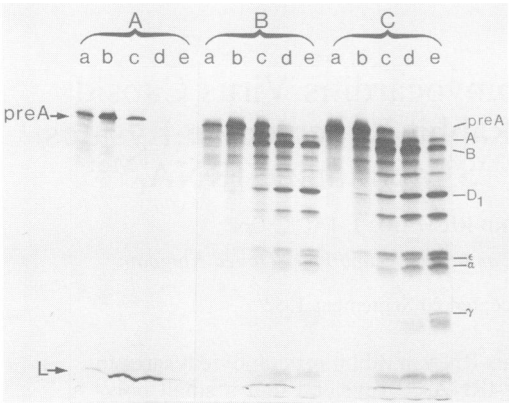

FIG. 2. Time course of labeling of EMC products in the presence of *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$. EMC RNA was translated at 47 μg/ml in the presence of (A) *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$ and 40 μM unlabeled L-methionine, (B) *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$ and 0.4 mCi of [^{35}S]methionine per ml, or (C) [^{35}S]methionine only. Lanes a through e correspond to samples incubated for 25, 40, 60, 100, and 180 min, respectively. All samples were run on the same gel, which was fluorographed for 60 h (panel A) or 5 h (panels B and C).

present showed that the presence of the formylated methionine tRNA did not interfere with the normal sequence of processing events and that substantial amounts of A and B had been formed by 60 to 100 min (Fig. 2). If the N-terminus of preA had been conserved on conversion to A and B, significant labeling of these two proteins would have been expected in the assay containing only *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$. This was clearly not the case, and only very weak labeling of proteins other than preA and L was observed (Fig. 2); this may arise either from premature termination of translation or from the transfer of labeled methionine from the methionine-tRNA$_f^{Met}$ contaminant to tRNA$_m^{Met}$.

These results are most readily interpretable in terms of the processing scheme shown in Fig. 1b. Since the cleavage site in preA is close to the N-terminus, it seemed possible that when EMC RNA is translated in a system which already contains the virus-coded protease necessary for secondary processing of capsid precursors (5, 8, 10), the cleavage might occur before completion of preA synthesis. This was tested by translating EMC RNA in a reticulocyte lysate system supplemented with unlabeled EMC translation products. The result was that A was the major capsid protein precursor labeled in the early stages of translation assays containing [^{35}S]methionine, and virtually no preA could be detected (Fig. 3). When *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$ was used in such experiments, the only protein labeled to a significant extent was the small cleavage product L (Fig. 3). We conclude that when EMC-coded processing en-

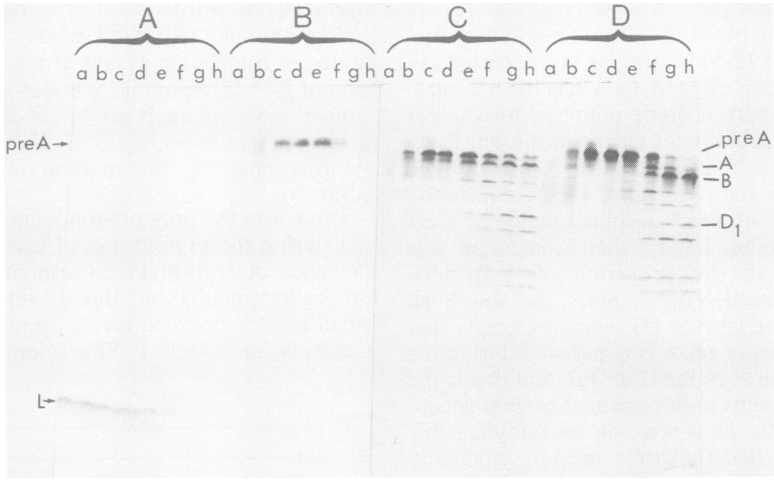

FIG. 3. Time course of appearance of EMC products synthesized in the presence of EMC protease. Unlabeled virus-coded proteins were made by translation of 60 μg of EMC RNA per ml in the absence of labeled amino acids for 100 min, and a control incubation lacking EMC RNA was run in parallel. Each was then diluted with an equal volume of fresh translation mix containing 60 μg of EMC RNA per ml, and these mixtures were incubated with *N*-formyl-[^{35}S]methionine-tRNA$_f^{Met}$ and either 40 μM L-methionine (A and B) or 0.2 mCi of [^{35}S]methionine per ml (C and D). Panels A and C correspond to assays supplemented with unlabeled EMC translation products; panels B and D are controls. Lanes a through h correspond to samples incubated for 11, 15, 20, 25, 30, 40, 60, and 80 min, respectively. All samples were run on the same gel, which was fluorographed for 36 h (panels A and B) or 6 h (panels C and D). Distortion on drying this gel has resulted in some skewing and compression of the right hand side, especially panel D.

zymes are present, the cleavage which removes L is a very rapid process which normally occurs before the synthesis of preA has been completed. This offers an explanation for the facts that translation of EMC RNA in extracts of infected Krebs ascites cells leads to formation of A rather than preA (4, 12) and the largest capsid precursor found in infected cells is normally A, although a polypeptide similar to preA has been seen in infected cells exposed to $ZnCl_2$ (1). It is also interesting to record that L decreases in amount during prolonged incubation of the reticulocyte translation system (Fig. 2 and 3) and is the only unstable small product we have noted in these studies.

The molecular weight of L should be equal to the difference in size between preA and A, for which estimates in the range of 8 to 12,000 have been given in different reports (4, 5, 9, 10). The protein described by Lawrence and Thach, which is almost certainly the same as L, was assigned a molecular weight of 12,500 (4). In our gel system, L migrates slightly slower than globin, which is indicative of a value of about 17,000; on the other hand, it migrates faster than G, which has been assigned a molecular weight of 15,000 (9, 10). These discrepancies probably result from the influence of amino acid composition on electrophoretic mobility in different gel systems, coupled with the distorting effect of the relatively large globin loading. Thus, the translation initiation site of EMC RNA is likely to be located some 300 to 500 nucleotide residues (depending on the exact size of L) nearer the 5′ end of the RNA than the site coding for the N-terminus of the capsid proteins δ and ϵ and the capsid precursor intermediates A, B, and D_1.

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