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Translation of Encephalomyocarditis Virus RNA in Reticulocyte Lysates: Kinetic Analysis of the Formation of Virion Proteins and a Protein Required for Processing

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In cell-free extracts derived from rabbit reticulocytes, encephalomyocarditis RNA can be translated completely, and the products can be processed extensively to give encephalomyocarditis virion proteins and several nonvirion proteins, including a genome-coded protein required for processing. The latter is probably a protease. Translation is very efficient. Under typical conditions, each EMC RNA is translated approximately eight times during a 3-h period. Kinetic analyses (time-course experiments, pulse-chase experiments, and pulse-stop experiments) have been used to determine the time of appearance of major products, and these times have been correlated with map positions. The gene for the putative protease is located near the middle of the genome downstream from the virion protein genes. Ribosomes can travel the length of encephalomyocarditis RNA within 30 min, but there is a delay in their progress along the RNA at some point soon after they traverse the region coding for virion protein precursors. This delay results in the accumulation of precursors for a period of about 10 min before the putative protease is made and virion proteins (ϵ, α, and γ) are released by proteolysis.

Encephalomyocarditis (EMC) virus is a member of the picornavirus group. In vivo studies indicate that EMC RNA translation starts mostly at a single initiation site and proceeds through almost the entire length of the genome. The synthesized protein is processed, at the nascent chain stage, into at least three primary products: among them, a capsid protein precursor, A, and the noncapsid proteins F and C. The intact polyprotein, containing A, F, and C, is detectable only under conditions in which cleavage of the synthesized protein is inhibited (3). The capsid protein precursor is cleaved to give proteins α, ϵ, and γ. In the final stage of virion maturation, after packaging of the viral RNA, ϵ is cleaved into proteins β and δ. F is a stable protein. Protein C is processed to yield protein D and then protein E (2, 4, 13). Fig. 1 shows the cleavage map (3).

Early attempts to translate picornavirus RNAs in vitro were not successful. The translation products in most cases consisted of a spectrum of peptides of various lengths whose existence was attributed to premature peptide chain termination. The first definitive identification of viral proteins synthesized in a cell-free system was by Villa-Komaroff et al. (17). They showed that in extracts derived from HeLa cells, poliovirus RNA directs the synthesis of proteins 1a, X, 1b, 2, and 4 (corresponding to EMC viral proteins A, F, C, D, and E, respectively). The capsid protein 1a was the major product under all conditions reported.

Studies of picornaviral RNA translation have been greatly facilitated by the procedure of Pelham and Jackson for treating cell-free extracts with nuclease to remove endogenous mRNA's (12). Svitkin and Agol, using nuclease-treated Krebs-2 ascites cell extracts, showed that a number of proteins synthesized in their system had electrophoretic mobility identical to that of proteins made in EMC-infected cells (15). They showed that in syntheses carried to completion (2.5 h), products co-electrophoresing with virion proteins α and γ were detectable. However, in a later study in which they analyzed products as a function of time, no virion proteins were evident during a 2-h period (16). Pelham showed that EMC RNA can be translated completely in rabbit reticulocyte extracts, and that processing of these products can be followed with time (11). He found that some of the processing is effected by a proteolytic activity resulting from the translation. In this study also, only a small proportion of precursors was cleaved. Recently we showed that polio RNA can be translated completely in rabbit reticulocyte extracts and that the products are extensively processed (14). In the same

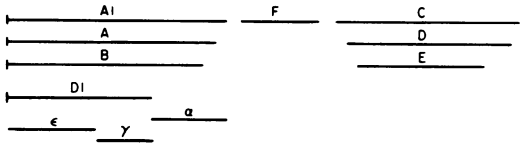

FIG. 1. *Cleavage map of the EMC viral proteins (modified slightly from reference 3 to include protein A1, a major protein found in in vitro translation).*

study we reported briefly that EMC viral RNA can be translated accurately in reticulocyte extracts, and, furthermore, the processing into virion proteins α, γ, and ε and nonvirion proteins D and E is extensive.

The translation efficiency of EMC RNA is about fourfold higher than that of polio RNA (14), and this fact along with the completeness of processing of the synthesized EMC products prompted us to study translation of its RNA further with the objective of gaining a better understanding of the posttranslational cleavage reactions. In this paper, we will demonstrate the progression of translation and of cleavage resulting in the appearance of precursors, a genome-coded proteolytic activity, intermediate cleavage products, and final cleavage products. We also demonstrate an apparent delay in the progress of ribosome along the RNA at a region after completion of the virion protein precursors but before completion of protein F and define a map location for the genome-coded proteolytic activity downstream from this obstruction. This provides experimental evidence for a novel mechanism by which EMC can temporally regulate expression of its genes.

MATERIALS AND METHODS

Virus and RNA. Propagation of EMC virus and RNA isolation were as previously described (6, 18).

Preparation of rabbit reticulocyte lysate and conditions for protein synthesis. Reticulocyte lysates were prepared according to the procedure previously described (14). The lysate was made mRNA dependent by treating with micrococcal nuclease according to a method slightly modified from that of Pelham and Jackson (12). After the lysate was thawed, creatine kinase (50 μg/ml), hemin (50 μM), Tris-hydrochloride (pH 8.2) (20 mM), potassium acetate (10 mM), staphylococcal nuclease (75 U/ml), and CaCl (1 mM) were added to the final concentrations indicated. A typical nuclease digestion mixture contained 183.5 μl of lysate, 4 μl of 1 M Tris-hydrochloride, 1 μl of 2 M potassium acetate, 2 μl of 5-mg/ml creatine kinase (in 50% glycerol), 2.5 μl of 4 mM hemin (in 90% ethylene glycol), 2 μl of 100 mM CaCl, and 1 μl of micrococcal nuclease at 15,000 U/ml. The mixture was incubated at room temperature for 10 min and the nuclease was inactivated by adding 4 μl of 100 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (neutralized with KOH).

A standard protein synthesis mixture (30 μl) contained 17.5 μl of the nuclease-treated lysate plus the following assay components: 10 mM creatine phosphate, 90 μM each of 19 unlabeled amino acids, 58 μg of calf liver tRNA per ml, 5 mM dithiothreitol, 0.6 mM magnesium acetate, 100 mM potassium acetate (including that added to the lysate), and [^{35}S]methionine (600 to 750 Ci/mmol) which was 0.8 μM for most experiments. Incubation was at 30°C for 3 h unless stated otherwise.

Electrophoresis. Conditions for treatment of samples and for slab gel electrophoresis were as described (14). Normally, 5-μl samples of the original reaction mixtures were analyzed. After electrophoresis, the gel was soaked in 400 ml of a solution containing 25% methanol, 10% acetic acid, and 1% glycerol for 4 h with two changes of solution. The gel was dried and exposed to a Kodak NS 54T no-screen X-ray film for 1 or 2 days.

Materials. [^{35}S]methionine was obtained from Amersham Corp. Staphylococcal nuclease and calf liver tRNA were obtained from Boehringer Mannheim Biochemicals. Sparsomycin was a gift of John Douros, Develpmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

RESULTS

General properties of the incorporation reaction. Translation of EMC RNA proceeds exceptionally well in rabbit reticulocyte lysates. Upon addition of EMC RNA, amino acid incorporation is readily detectable within 5 min and continues for about 3 h. In the range of 0 to 30 μg/ml, incorporation is proportional to RNA concentration and levels off thereafter.

Maximum incorporation occurs when magnesium acetate and potassium acetate are added to levels of approximately 0.6 mM and 100 mM, respectively, and depends slightly on the batch of reticulocyte lysate. We estimate an endogenous contribution of 0.2 mM Mg^{2+} and 50 mM K^+ so that maximum incorporation occurs at approximately 0.8 mM Mg^{2+} and 150 mM K^+. Incorporation is roughly halved when the concentrations of these ions are one-third or twice the optimum values.

From isotope dilution experiments we have determined that the concentration of methionine in our reaction mixture is about 10 μM. Under typical conditions, about 45% of this methionine is incorporated in 180 min when the concentration of EMC RNA (molecular weight = 2.6×10^6) is 30 μg/ml, i.e., 11.5×10^{-3} μM. Thus, approximately $4.5/(11.5 \times 10^{-3}) = 390$ methionine residues are incorporated per EMC RNA molecule. We estimate that the EMC polyprotein contains about 49 methionine residues. Thus each EMC RNA is translated approximately 390/49 = 8 times.

Enumeration of the products of translation and processing. Time-course analyses.

We have made analyses similar to those of Pelham (11) and confirm his observation that translation of EMC RNA in rabbit reticulocyte extracts results in the appearance of EMC-specific proteins, most of which can be identified with proteins synthesized in EMC-infected HeLa cells (2, 14). Both in vivo and in vitro most, if not all, of these proteins result from translation beginning at a single initiation site, followed by a series of proteolytic cleavages of the polyprotein product.

We have extended our analyses to establish the time of appearance of the synthesized EMC proteins, in particular the virion proteins ϵ, α, and γ and the viral RNA-coded protease. Figure 2 shows a radioautograph of a polyacrylamide

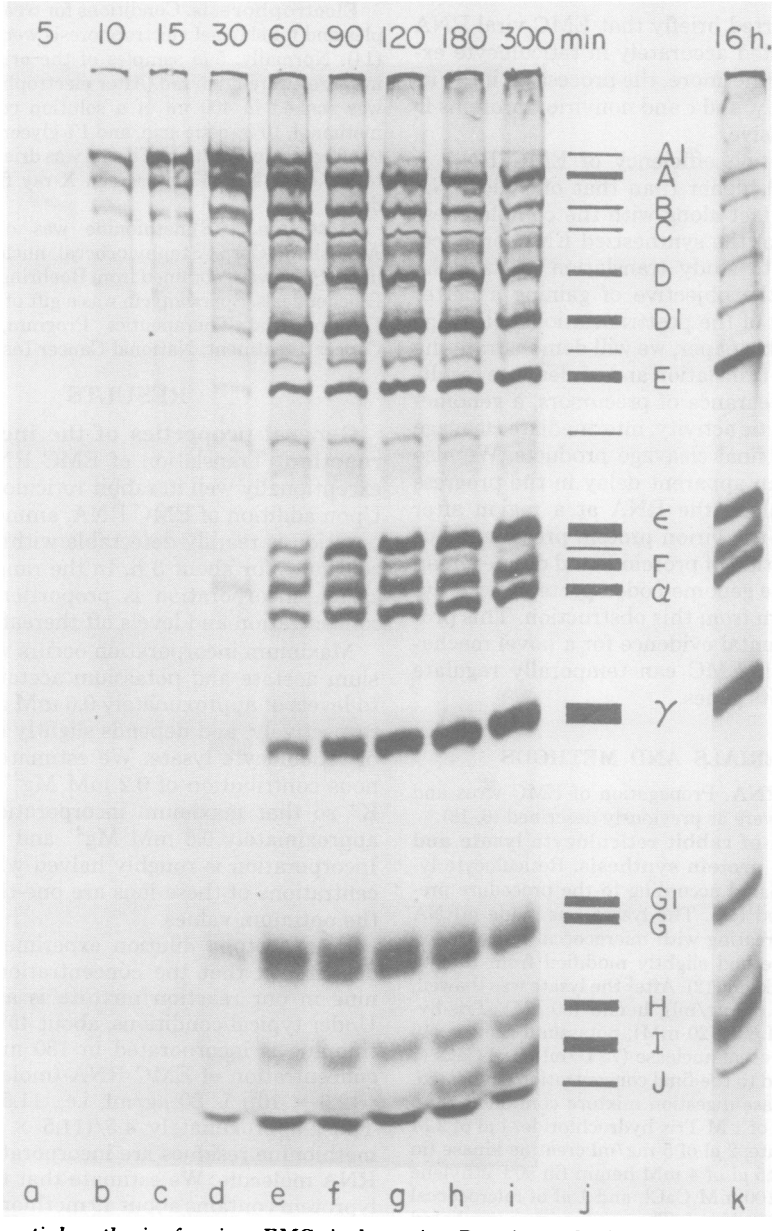

FIG. 2. *Sequential synthesis of various EMC viral proteins. Protein synthesis was carried out under normal incorporation conditions. Incorporation was stopped at the indicated times by adding EDTA and pancreatic ribonuclease. The samples were analyzed on sodium dodecyl sulfate-polyacrylamide gels. Lanes a through i correspond to samples incubated for 5, 10, 15, 30, 60, 90, 120, 180, and 300 min, respectively. Lane j is a diagram identifying the major band. Lane k corresponds to a sample incubated for 16 h.*

TABLE 1. *Apparent molecular weights calculated from band positions in Fig. 2*[a]

Band	Apparent mol wt
A1	108,000
A	100,000
B	92,700
C	87,500
D	78,600
D1	69,600
E	60,200
ϵ	40,600
F	36,900
α	34,800
γ	25,000
G1	(15,000)
G	(14,500)
H	(11,600)
I	(10,300)
J	(9,700)

[a] True and apparent molecular weights may differ substantially for proteins corresponding to bands G1, G, H, I, and J.

gel pattern of proteins synthesized (i.e., translated and processed) during various times after the addition of EMC RNA to reticulocyte lysates (lanes a to i), a diagram identifying the major bands (lane j), and a gel pattern after a 16-h incubation to allow near completion of processing (lane k). Table 1 lists the major bands together with their apparent molecular weight, calculated from their mobility assuming that protein A and protein γ have molecular weights of 100,000 and 25,000, respectively (9). The protein or proteins called preA by Pelham (11) we have redesignated A1 to conform to our nomenclature.

Returning to Fig. 2, it may be seen that the time of appearance and disappearance of the various bands is consistent with the map of Fig. 1. Lane a shows that within 5 min of the addition of messenger, bands A and B are already visible, although faint. Band A1 appears within 10 min and until 30 min is the strongest band. Bands F and C are visible within 30 min. Most other bands appear thereafter and change characteristically with time. After 16 h the only strong bands that remain are E, F, G, H, and I and the bands of EMC virion proteins ϵ, α, and γ. Clearly, proteolytic processing is almost complete.

It is evident that initial processing occurs on the nascent polyprotein to give A1, F, and C. From their early appearance it is probable that A and B are also derived directly from the nascent polyprotein. The intensity and time of appearance of bands D and E suggest that they may also derive directly from the polyprotein, but the eventual disappearance of C and D suggests that the processing route C $\rightarrow$ D $\rightarrow$ E exists.

Pulse-chase analyses. Although time course analyses are useful for determination of the quantity of a product as a function of time, they do not serve well in determining the progression of processing, because intermediate products are augmented by protein synthesis while they are depleted by the processing reactions. Processing and identification of intermediates can be followed more readily by pulse-chase analyses. Because of the high rate of translation, labeling times as short as 15 min give ample radioactivity for detailed studies. During the first 15 min, translation will be predominantly from the leftmost portion of the map of Fig. 1. This may be seen in Fig. 3, lanes a to e, which exhibits the proteins translated during that time and their processing over a period of 3 h. Incorporation proceeded for 15 min, at which time a large excess of nonradioactive methionine was added and incorporation and processing were continued. Since the changing intensities of the bands reflect redistribution of the label incorporated in the initial 15 min, processing can be followed independent of new incorporation. Lane a shows radioactive incorporation for 15 min followed by 15 min of further incubation. Comparison with lane c of Fig. 2 shows that the additional strong bands D1, G1, and H and moderately strong band α are present. Presumably these are cleavage products of proteins A1, A, and/or B. Band D1 is stronger than its complement band α because protein D1 is richer in methionine than is protein α (H. Yoshimura, Ph.D. thesis, University of Wisconsin, Madison, 1976). Lanes b to e show clearly that, with increased time of processing, proteins A1, A, and B decline in amount while virion proteins ϵ, α, and γ and protein G increase. Proteins D1 and G1 behave as intermediates, first increasing and then decreasing. The large amount of D1 remaining after 3 h suggests that its degradation is relatively slow.

Protein F is also visible though weak, and its amount remains constant. Probably translation into this region of the cistron has begun in the first 15 min and has continued with nonradioactive methionine during the chase period.

Lanes f to k (Fig. 3) show a corresponding experiment in which translation proceeded for 30 min in the presence of radioactive methionine and thereafter for various times with excess nonradioactive methionine. It may be seen that band F is now strong and, as before, invariant with time. Bands C, D, and E are present, indicating that translation into their portion of the mRNA has begun within the first 30 min. The decline in amount of C and D with time of chase is consistent with the notion of their processing into E, whose amount increases with time of chase.

Pulse-stop analyses. Although precursors of

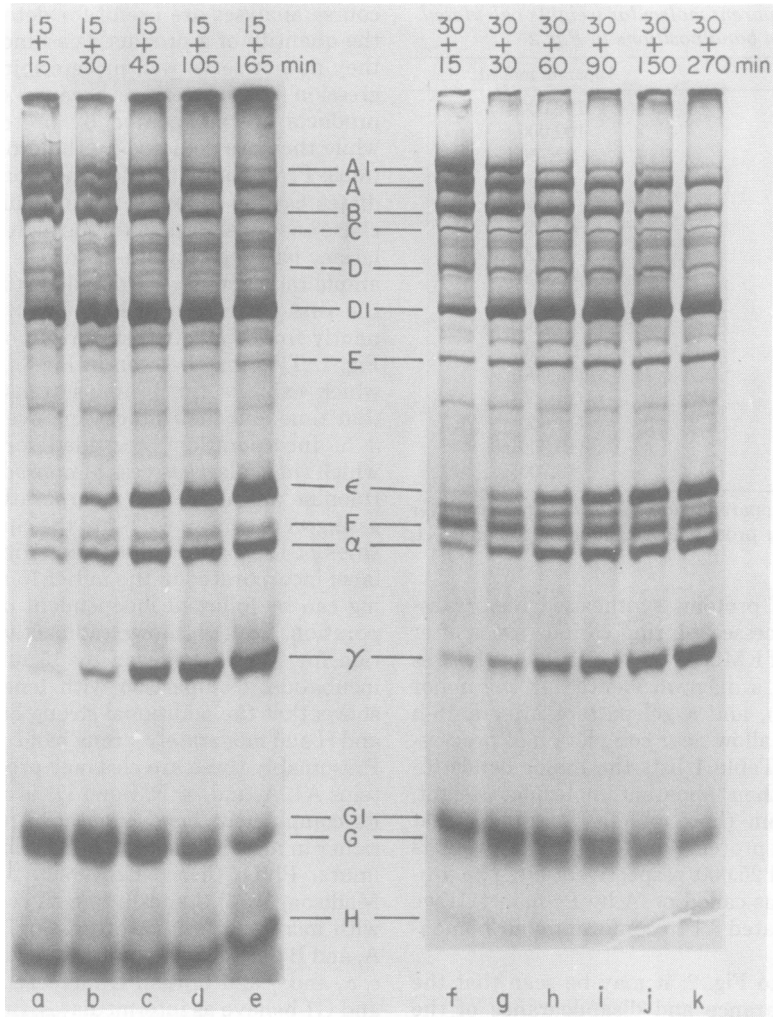

FIG. 3. *Pulse-chase analyses of the formation of EMC viral proteins. The viral proteins were labeled with [^{35}S]methionine for 15 min (lanes a to e) or for 30 min (lanes f to k). Nonradioactive methionine was then added to the reaction mixtures (60 μl) to 2 mM, and samples were withdrawn at the indicated times for analyses.*

the EMC virion proteins are evident in the first 10 min of translation, their processing does not commence until some 10 min later, suggesting that translation beyond these precursors is required for their processing. This feature of processing can be followed by terminating protein synthesis (as well as labeling) at various times so that processing can occur only by means of enzymes already present in the extracts. Such an experiment is shown in Fig. 4, which shows gel patterns where translation has been terminated at various times with the elongation inhibitor sparsomycin but proteolytic processing has been allowed to proceed during a long further incubation period (lanes a to g). For translation times shorter than 22 min, only bands A1, A, and B are produced, and these remain after prolonged further incubation. At 22 min the pattern changes abruptly (lane c) with the appearance of virion protein precursor D1, virion protein α, and protein G1. Another processing intermediate, E1, whose map position is unknown, also appears. Also barely visible are bands ε, γ, F, and G, which by 24 min are strong bands. Thus a new proteolytic activity appears after about 20 min of translation.

Figure 4 shows the emergence of band E at about 26 min, a weak band D at 30 min, but a minor band in the position of band C even at relatively early times. We suggest that transla-

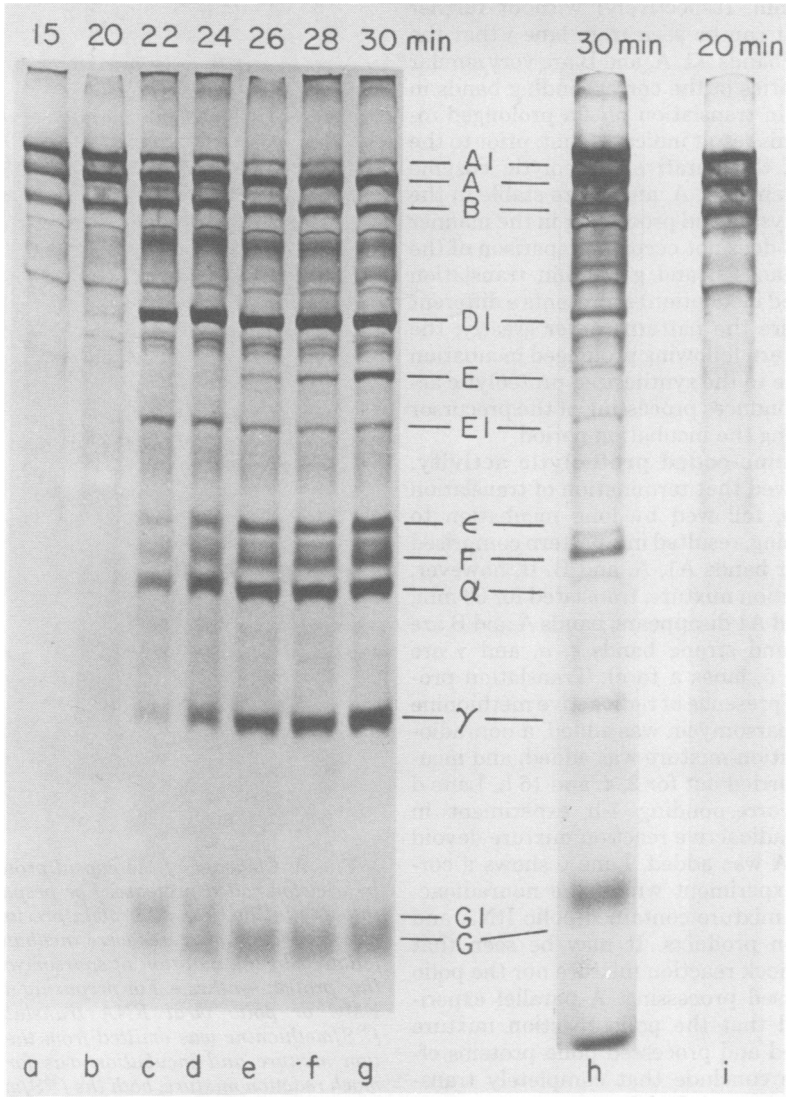

FIG. 4. *Pulse-stop analyses showing the effect of the length of incorporation on the viral protein cleavage reactions. Sparsomycin (200 μM) was added to the reaction mixtures at the indicated times to stop protein synthesis. Lanes a to g: incubation was continued for a total of 3 h after the sparsomycin addition. Lanes h and i: samples were withdrawn at the indicated times without further incubation.*

tion proceeds rapidly to generate B, A, and A1 very early, within 5 to 10 min of the time of messenger addition; then translation slows down markedly near F, traversing it at about 20 min, E at about 26 min, and D at about 30 min. The slowdown in the F region is consistent with studies we have in progress which show that translation traversing the F region requires additon of tRNA's to the reaction mixture, whereas translation to that region is much less affected by the tRNA addition.

The weak band D indicates that protein E can be produced directly from the nascent polyprotein prior to translation far enough to complete protein D. The presence of minor band C when band D has not yet appeared requires further study. Possibly its map position is incorrect; that is, in vitro band C may represent a protein different from in vivo band C. Possibly a small amount of rapid translation through the obstruction in region F is permitted, enough to see a trace of band C.

Lanes h and i show the gel patterns of samples taken right after the addition of sparsomycin (at

30 and 20 min, respectively) without further incubation. It can be seen from lane i that the intensities of bands A1, A, and B are very similar to the intensities of the corresponding bands in lane b (20-min translation plus a prolonged incubation). This result indicates that, prior to the formation of the putative proteolytic enzyme activity, proteins A1, A, and B are stable in the reticulocyte lysate and processing in the manner A1 → A → B does not occur. Comparison of the patterns of lanes h and g (30-min translation plus prolonged incubation) represents a different situation. Here the patterns differ greatly; the different pattern following prolonged incubation is attributable to the synthesized proteolytic activity, which induces processing of the precursor proteins during the incubation period.

EMC genome-coded proteolytic activity. Figure 4 showed that termination of translation after 20 min, followed by long incubation to allow processing, resulted in a pattern comprised of only major bands A1, A, and B. If, however, a second reaction mixture, translated for 60 min, is added, band A1 disappears, bands A and B are diminished, and strong bands ϵ, α, and γ are evident (Fig. 5, lanes a to c). Translation proceeded in the presence of radioactive methionine for 20 min, sparsomycin was added, a nonradioactive translation mixture was added, and incubation was carried out for 2, 4, and 16 h. Lane d shows the corresponding 4-h experiment in which a nonradioactive reaction mixture devoid of EMC RNA was added. Lane e shows a corresponding experiment where the nonradioactive reaction mixture contained polio RNA and its translation products. It may be seen that neither the mock reaction mixture nor the polio products caused processing. A parallel experiment showed that the polio reaction mixture had translated and processed polio proteins effectively. We conclude that completely translated and processed EMC extracts contain a proteolytic activity that can generate EMC virion proteins from precursors. Presumably this activity is due to a genome-encoded protease, but it is also possible that the synthesized protein in some way modifies a protease already existing in the reticulocyte extracts. In any event our results show that the synthesized protein is required for processing.

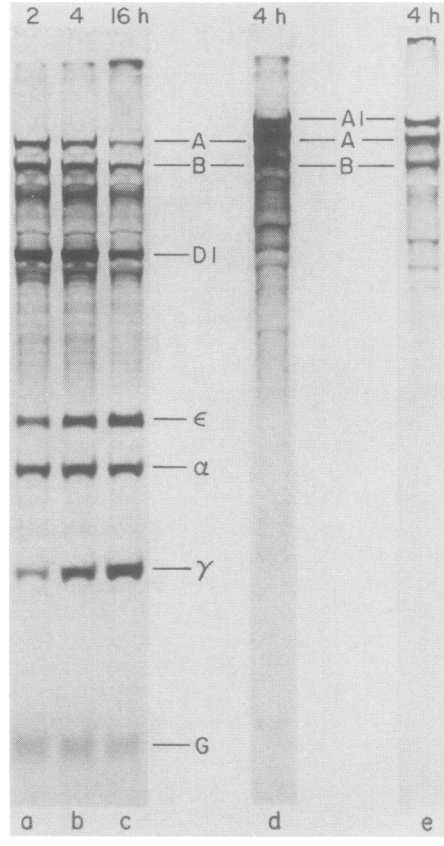

FIG. 5. *Cleavage of the capsid protein precursors by a virion-coded protease. For preparing [^{35}S]methionine-labeled capsid protein precursors, incorporation reaction mixtures were incubated for 20 min, followed by the addition of sparsomycin to stop further protein synthesis. For preparing nonradioactive EMC or polio viral RNA translation products, [^{35}S]methionine was omitted from the normal reaction mixture and incubation was for 1 h. For the mock reaction mixture, both the [^{35}S]methionine and the viral RNA were omitted. Lanes a to c: 1 volume of the radioactive precursor-containing reaction mixture was mixed with 1 volume of the reaction mixture containing nonradioactive EMC viral RNA products and incubated for 2 h (lane a), 4 h (lane b), and 16 h (lane c). Lanes d and e: the radioactive precursors were mixed with the mock reaction mixture (lane d) or the polio viral protein-containing reaction mixture (lane e) and incubated for 4 h.*

DISCUSSION

Our results suggest that, in rabbit reticulocyte extracts, translation of EMC RNA proceeds as follows. Translation of the left half of the RNA proceeds rapidly. This portion of the resulting nascent protein has three sites susceptible to proteolytic cleavage by reticulocyte enzymes to yield the proteins A1, A, and B. These proteins represent translation from the beginning of the cistron to the three susceptible sites and thus differ from each other only in their carboxyterminal region. Once generated, these proteins are resistant to further cleavage by reticulocyte enzymes so that conversion of A1 to A and B is not detectable, even after prolonged incubation

(Fig. 4). Translation then markedly slows in traversing the middle portion of the RNA. The existence of some kind of mechanism delaying polypeptide chain elongation in a specific region downstream of A1 seems clear on two grounds. First, we observe completion of A1 (108,000 daltons) within 10 min, whereas completion of significant amounts of F (37,000 daltons) requires much longer than the additional 4 min predicted if elongation beyond the end of A1 were equally fast. Second, poliovirus RNA, which is very similar in size to EMC and directs synthesis of analogous products, can be completely translated under identical conditions within about 10 min (14). Comparison of the results of polio RNA translation with EMC RNA translation as presented here seems to indicate that the blocking mechanism, whatever it is, is not universal among picornaviruses. We do not understand why these two apparently similar viruses should differ in this respect, but the ability to make relatively pure uncleaved capsid precursors in the case of EMC is experimentally very convenient and has allowed us to make a much more detailed analysis of the cleavage process than was possible with polio.

Once translation proceeds beyond this block, protein F appears and also the members of the C, D, E family. Proteolytic cleavage of the capsid protein precursors A1, A, and B is correlated with the appearance of the proteins coded by the right-hand half of the genome, one of which is clearly responsible for the proteolytic activity. Our results do not identify which of these it is. Preliminary experiments (unpublished) in collaboration with Ann Palmenberg indicate that the putative protease is not protein F or protein G but may well be a protein we designate P22 that comigrates with virion protein γ under our conditions of electrophoresis (4). This is in partial agreement with the finding of Lawrence and Thach that virion protein γ has proteolytic activity (7).

We should reiterate at this point one observation that may not fit the scheme just outlined, namely, the early appearance in small amounts of a band in the position we identify with protein C. Work is in progress to examine the relationship of this early-appearing "C" band to the others by tryptic peptide mapping.

It can be seen from the data that, in contrast to the stability of A1, A, and B in the absence of the viral protease, label can always be chased from C and D into E on prolonged incubation even after translation has been stopped so that only the processing of released polypeptides is being observed. Either the reticulocyte or the putative viral protease could be responsible for these cleavages, since both are present by the time C is synthesized. Small fragments expected as by-products in all these cleavage reactions remain unidentified. Therefore we cannot determine, for example, whether A1, A, and B may all be cleaved to give D1 and α and, if so, what is left over in each case.

On surveying the entire process as we observe it, one is particularly struck by the long delay encountered by ribosomes downstream of polypeptide A1. One would like to know, first, does this happen in vivo, and, if so, how is it accomplished and what, if any, purpose does it serve? If it happens in vivo, it should lead to a transient overproduction of capsid proteins and their precursors midway through exponential growth, compared to the equimolar yields of products expected if all ribosomes have run off the mRNA. There have indeed been some reports that capsid proteins are made in excess in cardiovirus-infected cells (8, 10), although these observations may be partly due to uneven labeling of the various products (11) or to undercounting the translation frequency of regions downstream from the coat protein (13). With regard to mechanism, we have preliminary evidence indicating that an addition of exogenous tRNA's to the reaction mixture increases synthesis past the block. Therefore a cluster of codons requiring minor isoaccepting tRNA species is one candidate for the block, possibly together with a highly structured region of the RNA. We should point out that the polycytidylic acid tract known to exist in EMC RNA (1) cannot be responsible since it is too close to the 5′ end of the RNA to be located downstream of A1 (5). Finally, as far as function is concerned, it is probably too early to make good suggestions. However, the long lag period in which capsid precursors are stable before processing begins does raise the possibility that in synchronous infection the capsid protein precursor has an early function separate from its later structural role.

ACKNOWLEDGMENTS

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