DEGRADATION OF VIRAL MESSENGER RNA BY ENZYMES FROM ESCHERICHIA COLI

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Polypeptide synthesis by cell-free extracts from E-coli is highly stimulated by the addition of viral RNA (Nirenberg et al.; Tsugita et al.; Haselkorn et al., 1963; Nathans et al., 1962). In this laboratory the messenger function of plant viral RNA has been the subject of a number of investigations (Voorma et al., 1964; Voorma et al., in press). It has been demonstrated (Voorma et al., in press) that the association of varying numbers of isolated 70S ribosomes with turnip yellow mosaic virus RNA (TYMV-RNA) may occur at 0° under appropriate conditions and in the complete absence of protein synthesis. This association reaches a saturation point at about 10-15 ribosomes, added per messenger molecule. Polypeptide synthesis is initiated upon addition of amino acids, soluble enzymes and cofactors to the polysomal aggregates thus formed. Under these conditions viral RNA is rapidly inactivated, however. Presently the degradation of the messenger is investigated in more detail and some of the findings are reported in this paper.

Cell-free extracts from E-coli, deprived of their endogenous messenger RNA, were prepared according to the slightly modified procedure of Nirenberg et al. (1961), as described previously (Voorma et al., in press). These extracts were centrifuged at 36.000 rpm in the Spinco ultracentrifuge for 3.5 hours, yielding a ribosomal pellet A and a supernatant B. The pellet A was resuspended in standard buffer, containing 0.01 M Tris-HCl, pH 7.8; 0.01 M magnesium acetate; 0.06 M KCl and 0.006 M mercaptoethanol and recentrifuged for another 3.5 hours. The final ribosomal fraction was designated K^{+}-ribosomes, as it was prepared in media con-

taining K^+. Messenger degradation has been followed in either of two ways:

a by studying inactivation of the messenger. TYMV-RNA was preincubated with either ribosomes or soluble enzymes for various periods of time. Subsequently the reaction mixture was supplemented with the components required for protein synthesis and incubated for another 30 min. at 37° (cf. legend of figure 1).

b by studying the appearance of acid soluble nucleotides during degradation.

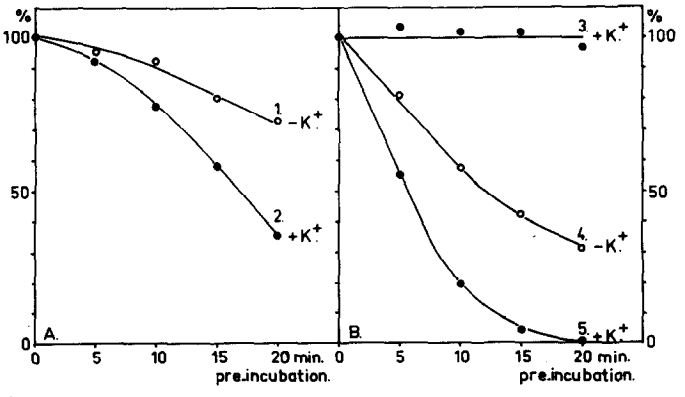

Figure 1. Inactivation of TYMV-RNA by K^+-ribosomes and the soluble fraction. Curve 1: a mixture (0.5 ml), containing in µmoles 50 Tris-HCl, pH 7.8, 9 magnesium acetate, 3 mercaptoethanol, 55 µg TYMV-RNA and 710 µg K^+-ribosomes was preincubated at 37°. Afterwards the reaction mixture was rapidly cooled, supplemented with 0.2 ml 105,000 x g supernatant, KCl and necessary cofactors, yielding a volume of 1.5 ml, which contained in µmoles 150 Tris, 27 magnesium acetate, 81 KCl, 9 mercaptoethanol, 1 ATP, 5 phosphoenol pyruvate, 0.12 GTP, 20 µg pyruvate kinase, 2 mµmoles each of ^{14}C-labeled threonine, lysine, phenylalanine, leucine and serine and 2 mµmoles each of 16 non-labeled additional amino acids. After incubation at 37° for 30 minutes, the radioactivity of the TCA insoluble fraction, washed and prepared according to conventional methods, was determined and expressed as percentage of the activity without preincubation.
Curve 2: preincubation with K^+-ribosomes in the presence of 27 µmoles KCl.
Curve 3: as curve 2, but 0.15 ml 105,000 x g supernatant D instead of ribosomes. Following preincubation K^+-ribosomes and cofactors were supplemented.
Curve 4: as curve 1, but 0.05 ml of resuspended pellet C (absorbancies at 230, 260 and 280 mµ: 670, 320 and 219 resp.) instead of ribosomes. Following preincubation K^+-ribosomes, supernatant D and cofactors were supplemented.
Curve 5: as curve 4, but 27 µmoles KCl during preincubation.

Preincubation of K^+-ribosomes with TYMV-RNA in a ratio of 10 ribosomes per RNA molecule results in inactivation of the messenger (figure 1, curve 1), which is enhanced by K^+ (curve 2). The supernatant B can be freed of inactivating enzymes by sedimentation at 36.000 rpm for 18 hours, yielding a pellet C containing all degradative activity (curves 4 and 5) and a supernatant D virtually devoid of this activity (curve 3). Inclusion of phosphate in the medium does not enhance messenger inactivation by all the fractions studied in figure 1. Apparently polynucleotide phosphorylase is either absent or non-detectable under our conditions.

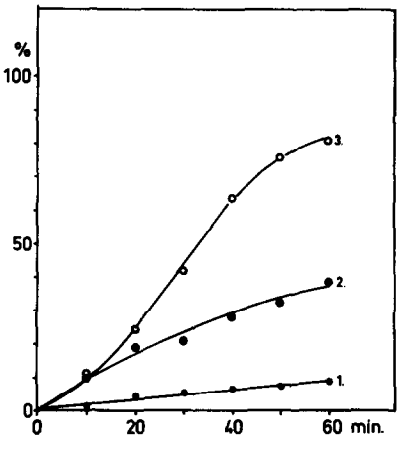

Figure 2. Curve 1: a mixture (1 ml), containing 76 µg E-coli ribosomal RNA, 456 µg NH_4^+-ribosomes, 50 µmoles Tris-HCl, pH 7.8, 75 µmoles KCl, 18 µmoles magnesium acetate, and 6 µmoles mercaptoethanol, was incubated for 0, 10, 20, 30, 40, 50 and 60 min., cooled and precipitated with 0.5 ml 5% perchloric acid and 0.25% uranyl acetate. After 10 min. another 1.5 ml 5% PCA was added. The precipitate was removed and the absorbancy at 260 mµ of the supernatant was read. Increase in absorbancy is expressed as percentage of the increase after alkaline hydrolysis.
Curve 2: 57.4 µg TYMV-RNA and 456 µg NH_4^+-ribosomes, for further details see curve 1.
Curve 3: 61 µg ribosomal RNA and 328 µg K^+-ribosomes, for further details see curve 1.

In an attempt to eliminate residual degradative activity from K^+-ribosomes the ribosomal pellet A was sedimented through a layer of 0.5 M NH_4Cl in 10% sucrose, 0.01 M Tris-HCl, pH 7.8, 0.01 M magnesium

acetate and 0.006 M mercaptoethanol. The pellet was resuspended in standard buffer and dialysed against this buffer for 18 hours. Ribosomes thus treated, were designated NH_4^+-ribosomes. Sucrose-gradient centrifugation revealed that they associate equally well with viral RNA as K^+-ribosomes. Similar experiments as in figure 1 showed that NH_4^+-ribosomes are lower in degradative activity than K^+-ribosomes but not completely free of it. The degradative capacity of the two classes of particles also differs qualitatively as becomes apparent from their effect of E-coli ribosomal RNA (figure 2). K^+-ribosomes rapidly degrade ribosomal RNA yielding about 80% of acid soluble nucleotides in 60 min. (figure 2, curve 3). By contrast NH_4^+-ribosomes hardly affect ribosomal RNA (curve 1) but nevertheless degrade TYMV-RNA (curve 2). It has been demonstrated by Okamoto and Takanami (1963) that ribosomal RNA does not associate with ribosomal particles under conditions, comparable with ours. It is possible therefore that the differential behaviour of NH_4^+-ribosomes towards ribosomal and viral RNA respectively is related to the different degree of association between these ribosomes and the two types of RNA. K^+-ribosomes on the other hand, which exert a strong degradative action on either RNA, may contain degrading enzymes which are released from the 70S particles during resuspension and/or incubation and thus become accessible to ribosomal RNA. Apparently sedimentation through 0.5 M NH_4Cl removes the latter enzymes from the ribosomes.

It has been observed previously that cell-free extracts prepared from E-coli stop incorporation of amino acids after 24-30 min. of incubation (Voorma et al., in press). NH_4^+-ribosomes programmed with TYMV-RNA continue incorporation up to about 100 min., when combined with supernatant D, radioactive amino acids and cofactors (figure 3, curve 1). In the absence of TYMV-RNA this incorporation is negligible (figure 3, curve 2). Release of radioactive polypeptides from NH_4^+-ribosomes also proceeds for about 100 min. (figure 4, curve 1).

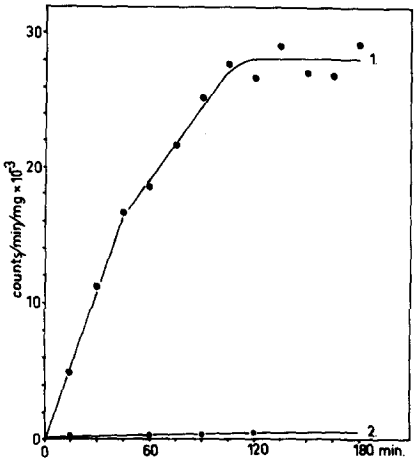

Figure 3

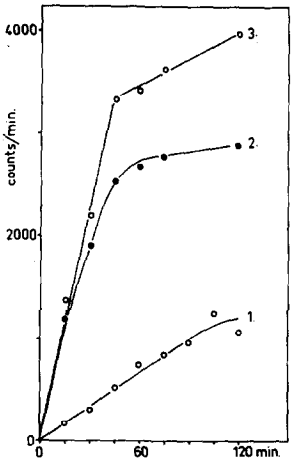

Figure 4

Figure 3. Time course of amino acid incorporation.
Curve 1: incubation mixture (1 ml), contained 73 µg TYMV-RNA and 470 µg NH_4^+-ribosomes (ribosome/TYMV-RNA ratio 5), 0.2 ml supernatant D, 50 µmoles Tris-HCl, pH 7.8, 18 µmoles magnesium acetate, 75 µmoles NH_4Cl, 6 µmoles mercaptoethanol, 1 µmole ATP, 0.12 µmole GTP, 5 µmoles phosphoenol pyruvate, 20 µg pyruvate kinase and 4 µmoles each of a mixture of labeled and non-labeled amino acids (see legend of figure 1). After incubation at 37° for the times indicated one volume of 10% TCA was added. Radioactivities were expressed as counts per min. per mg protein.
Figure 4. Amino acid incorporation into soluble and ribosome-bound polypeptides.
Incubation mixture (1 ml), contained 79 µg TYMV-RNA and 510 µg NH_4^+-ribosomes, for further details see figure 3, curve 1. Following incubation the reaction mixtures were centrifuged for 4.5 hours at 105.000 x g, the radioacties of both ribosomal pellet (curve 2) and supernatant (curve 1) were measured and expressed as total counts incorporated. Curve 3 is a summation of curves 1 and 2.

The loading of the ribosomes with nascent polypeptide chain, however, levels off after 45-60 min. (figure 4, curve 2). Consequently total incorporation (curve 3) starts to run parallel to the release curve after 45 min. (cf. the shape of figure 3, curve 1). After 100 min. of incubation total incorporation comes to a complete stop (figure 3), but is resumed when fresh viral RNA is supplied. Apparently degradation of the messenger has become so extensive at that time that further release of polypeptide chains from ribosomal aggregates is fully blocked. It is feasible that scission of the messenger chain is the cause of such a block, which

would then suggest that release of polypeptides is only possible at the natural end of each cistron. Alternatively one or more ribosomes attached to the messenger may become immobile during incubation and block the movement of the others.

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