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THE ROLE OF RIBOSOMES IN STABILIZING BACTERIOPHAGE T4 DEOXYNUCLEOTIDE KINASE mRNA IN VITRO

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SUMMARY

In the present investigation, an approach toward defining the role of ribosomes in stabilizing functional messenger RNA in cell-free extracts is described. The data presented show that initiation of protein synthesis is necessary for maximal functional stability of bacteriophage T4 deoxynucleotide kinase mRNA *in vitro* and suggest that much of the stability is attained by interaction of the deoxynucleotide kinase mRNA initiation site with a 30S ribosomal subunit. Data is also presented which suggest that any of several *E. coli* ribonucleases could serve as a messenger ribonuclease *in vivo*.

INTRODUCTION

That ribosomes can protect procaryotic mRNA from both chemical and functional decay *in vivo* has been well documented (1-4). Increased stability of mRNA is usually observed when ribosomes are frozen along an mRNA molecule, suggesting that the protection is due either to ribosomes covering ribonuclease sensitive sites or altering the secondary structure of these sites relative to a ribosome-free mRNA molecule. Recently, using antibiotic inhibitors of protein synthesis *in vivo*, it was shown that specific T4 mRNAs have nuclease sensitive sites in different portions of their respective mRNA molecules. For example, the nuclease sensitive site appears to be in or near the initiation site of the T4 deoxynucleotide kinase (EC 2.7.4.4) mRNA and distal to the initiation site of the T4 α-glucosyl transferase (EC 2.4.1.2) mRNA (3,4).

Methods are now available for determining whether ribosome interaction with a specific mRNA *in vitro* affects its functional stability. For example, it should be possible to determine the functional decay rate of a specific message in the presence of endogenous ribonuclease *in vitro* either

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when bound to ribosomes or ribosome free, as long as that message can be translated *in vitro*. In the present report, we use this approach to show that initiation of protein synthesis is required for maximal bacteriophage T4 deoxynucleotide kinase mRNA stability *in vitro*.

MATERIALS AND METHODS

Bacteria, bacteriophage, and conditions of infection. *Escherichia coli* B207 and bacteriophage T4 amBL292 were used. Cells were cultured in a glucose-salts medium supplemented with 0.3% casamino acids (5). Strain B207, which is non-permissive for amBL292 was infected at a multiplicity of 5 phage per cell.

Isolation of RNA. RNA was extracted from cells 12 min after infection at 37°. AmBL292 infected *E. coli* B207 synthesize prereplicative T4 mRNA exclusively (6). AmBL292 RNA isolated 12 min. after infection (12 min amBL292 RNA) has high deoxynucleotide kinase (hereafter called kinase) mRNA activity and was used as the template for the *in vitro* studies.

Preparation of ribosomes, S-100 and initiation factors. An undialyzed extract of B207 cells was prepared as previously described (7). The ribosomes were collected by centrifugation at 100,000 x g for 1.5 hr, and washed twice with ribosome buffer (0.01 M Tris-HCl, 0.01 M MgAc, 0.03 M KCl, pH 7.5, containing 6 mM mercaptoethanol, 5% glycerol and 1.25 mM dithiothreitol). The top two thirds of the supernatant from the first 100,000 x g centrifugation is the S-100 fraction and was dialyzed against ribosome buffer for 3 hr. Crude initiation factors were prepared from a 1 M NH_4Cl wash of ribosomes as previously described (8). All fractions were divided into 0.5 ml aliquots, frozen in liquid nitrogen, and kept at -15°. They maintained optimal activity for about one month.

Formation of Kinase mRNA-initiation complexes. As used here, a kinase mRNA-initiation complex consists of a ribosome bound to the initiation site of the message. The conditions used for formation of kinase mRNA-initiation complexes were similar to those described previously (8). Each complete reaction tube contained in 0.2 ml, 60mM Tris-HCl (pH 7.5), 8mM dithiothreitol, 72 mM NH_4Cl, 7.5 mM $MgCl_2$, 4.8 ug creative phosphokinase, 10mM creative phosphate, 3mM ATP, 0.3 mM GTP (pH 7.0), 100 ug *E. coli* stripped tRNA, 0.6mM folinic acid, 0.7mM methionine, dialyzed S-100 (375 ug protein), salt-washed ribosomes (167 ug), initiation factor fraction (25 ug protein) and 12 min. amBL292 RNA (50 ug). Under these conditions, 4.1×10^{-2} n moles of methionyl-tRNA were bound to ribosomes in 20 min at 37° (data not shown). In the absence of initiation factors only 4.1×10^{-3} n moles of mehtionyl-tRNA were bound to ribosomes (data

not shown). In the absence of S-100 no methionyl-tRNA was observed bound to ribosomes (data not shown).

Translation Conditions. Conditions suitable for initiation were not adequate for kinase mRNA translation because the concentration of S-100 was limiting and no amino acids except methionine were added. Doubling the concentration of S-100 in itself was not sufficient for observable kinase mRNA translation but when, in addition, a complete mixture of amino acids(0.14 mM, final concentration of each) was added, enough kinase was translated to convert 7.0 n moles of (^{14}C)-Hydroxymethyl deoxycytidylate monophosphate (900 CPM/n mole) to the diphosphate and triphosphate under conditions of the assay (7). In the absence of 12 min. amBL292 RNA 0.2 n moles of the di- and triphosphates were formed and this value was subtracted from each experimental value.

Chemicals. ^{14}(C)-formaldehyde (12 Ci/mole) and L-methyl - ^{3}(H)-methionine (14.6 Ci/mole) were purchased from New England Nuclear Corp. ATP, GTP, phosphoenolypyruvate, pyruvate kinase, dCMP, tetrahydrofolic acid (grade III), dithiothreitol, 2-mercaptoethanol and bovine pancreatic ribonuclease were purchased from Sigma. E. coli B tRNA was purchased from GIBCO. L-amino acids were purchased from Calibiochem and folinic acid was a gift from Lederle Laboratories.

RESULTS

The functional stability of kinase mRNA in vitro. Since we are able to determine the ability of 12 min amBL292 RNA to serve as a template for the in vitro synthesis of functional kinase, the loss of this ability is a measure of the functional stability of the kinase messenger under a specific set of conditions. In these experiments, the effect of three cell fractions on in vitro kinase mRNA stability was determined.

Twelve min amBL292 was incubated together with either salt-washed ribosomes, initiation factors, or the S-100 fraction alone and in all combinations of two and three fractions together. The functional half-life of kinase mRNA under each of these conditions was then determined as described in the legend to Table 1. Each of the cell fractions contained endogenous nuclease activity which degraded kinase messenger (Table 1, A). When any one of the three fractions necessary for initiation of protein synthesis was omitted from reaction mixtures, the rate of kinase mRNA decay was slower than expected if the nuclease activities in the fractions were strictly additive (Table 1, B). However, the least deviation from additivity of nuclease activities occured in the absence of ribosomes (i.e. in the presence of S-100 and initiation factors whereas the most significant deviation from additivity of nuclease activities

TABLE 1. CONDITIONS THAT AFFECT THE IN VITRO STABILITY OF KINASE mRNA

Components of the in vitro system incubated with kinase mRNA	Observed $T_{\frac{1}{2}}$ (min)	Expected $T_{\frac{1}{2}}$ (min)	Ratio of Observed/Expected
(A) None	∞	---	---
Initiation factors	5.2	---	---
Dialyzed S-100	7.2	---	---
Salt-washed ribosomes	12.5	---	---
(B) Initiation factors + dialyzed S-100	4.5	3.0	1.5
Salt-washed ribosomes + Dialyzed S-100	8.4	4.6	1.8
Salt-washed ribosomes + Initiation factors	9.2	3.7	2.5
(C) Salt-washed ribosomes + Initiation factors + dialized S-100	13.5	2.4	5.6

(A) To determine the relative amounts of ribonuclease associated with salt-washed ribosomes (167 ug), initiation factors (25 ug) and S-100 (375 ug protein), each fraction was incubated for 0, 10, and 20 min with 12 min amBL 292 RNA (50 ug/0.2 ml reaction mixture). The relative amounts of functional kinase mRNA remaining at each time was determined by translating the remaining mRNA as described in Materials and Methods. Each reaction tube had its own control in which all conditions were identical except that 12 min amBL292 RNA was omitted during the initial incubation period and then added at the time of translation. The percent kinase mRNA remaining at any time for any reaction tube was then determined relative to its control. Functional kinase mRNA decay was first order as observed in vivo (3,4) and the half-life of decay ($T_{\frac{1}{2}}$) under each condition was determined.

(B) As described in (A) except that components were added two at a time. Expected half-lives were calculated assuming that the nuclease activities in the components are additive. Since the rate constant of kinase mRNA decay of any single component is Log $2/T_{\frac{1}{2}}$ where $T_{\frac{1}{2}}$ is the observed half-life of decay in response to that component, the expected half-life of kinase mRNA decay in response to any two components is Log 2 divided by the sum of the rate constants of each component.

(C) As described in (A) except that all components were added. The expected half-life of kinase mRNA was calculated as Log 2 divided by the sum of the three individual rate constants of mRNA decay.

TABLE 2. EFFECT OF BOVINE PANCREATIC RIBONUCLEASE (BPR) ON THE STABILITY OF KINASE mRNA-INITIATION COMPLEXES

Reaction Mixture	Observed $T_{\frac{1}{2}}$ (min)	Expected $T_{\frac{1}{2}}$ (min)	Ratio of Observed/Expected
Kinase mRNA + Salt-washed Ribosomes	15.0	---	---
Kinase mRNA + BPR	6.3	---	---
Kinase mRNA-Initiation Complexes	15.1	---	---
Kinase mRNA + Salt-washed Ribosomes + BPR	5.3	4.4	1.2
Kinase mRNA-Initiation Complexes + BPR	11.5	4.4	2.6

Experiments were performed and expected half-lives calculated as described in the legend to Table 1. BPR was used at a final concentration of 5.0×10^{-5} ug/ml.

in a two fraction system occured when messenger was incubated in the presence of initiation factors and ribosomes (i.e. in the absence of S-100). The latter incubation condition allows binding of a 30S ribosomal subunit to the initiation site of an mRNA molecule (9, 10). Conditions which allowed maximal initiation of protein synthesis (Table 1, C), resulted in an observed kinase mRNA decay rate less than five times that expected from the amounts of nuclease activity present in the reaction mixtures (Table 1, C).

Effect of bovine pancreatic ribonuclease (BPR) on the stability of kinase mRNA in vitro. The interaction between a ribosome and a kinase mRNA molecule could alter the message such that it becomes resistant to the action of a specific E. coli ribonuclease or to the action of any of a number of different ribonucleases. To determine which of these possibilities is more likely, reaction mixtures containing kinase mRNA, ribosomes, initiation factors, and S-100 (kinase mRNA-initiation complexes) and reaction mixtures containing kinase mRNA and ribosomes, but no initiation factors or S-100 (free kinase mRNA) were incubated in the presence and absence of BPR (final concentration, 5.0×10^{-5} ug/ml). The functional half-lives of kinase mRNA in the presence or absence of BPR was determined as described in the legend to Table 1. As illustrated in Table 2, kinase mRNA-initiation complexes are about two and a half times as resistant to the action of BPR as expected, based on its activity on ribosome-free kinase mRNA.

DISCUSSION

In the present investigation, we have described a cell-free system in which kinase mRNA-initiation complexes are functionally stable relative to ribosome-free kinase mRNA (Table 1). While we believe we have limited ribosome-kinase mRNA interaction to the initiation site by using a minimal amount of S-100 and leaving out all amino acids but methionine (see Materials and Methods), we cannot be sure that ribosomes do not move slowly beyond the initiation site in our system. This is because the small amount of S-100 required for N-formylmethionyl tRNA synthesis may contain enough aminoacyl-tRNA for some limited ribosome movement. However, a significant increase in kinase mRNA stability over that expected is also observed when messenger is incubated in the presence of both initiation factors and ribosomes, but in the absence of S-100 (Table 1). Under these conditions, kinase mRNA-ribosome interaction is limited to the binding of 30S ribosomal subunits to messenger initiation sites (9,10). Together these data suggest that the initiation site contains a nuclease sensitive site which determines the stability of the kinase mRNA *in vitro*. This conclusion agrees with our previous work which indicated that a ribosome frozen in the initiation site for protein synthesis is the only requirement for kinase mRNA stability *in vivo* (3,4). Since a ribosome covers approximately 40 nucleotides (11,12) and since the kinase mRNA is between 800 and 1200 nucleotides long (13,14), it is possible that as little as 3 to 5% of the kinase messenger may be uniquely sensitive to nuclease attack.

The nuclease sensitive site on the kinase message does not appear to be uniquely sensitive to a specific *E. coli* ribonuclease. That is, kinase mRNA-initiation complexes are resistant to the action of both the *E. coli* ribonuclease(s) present in the cell-free system (Table 1) and a completely unrelated endoribonuclease, bovine pancreatic ribonuclease (Table 2). *E. coli* contains several ribonucleases (15, 16). However, mutants which are deficient in any one of these enzymes either functionally degrade mRNA at the same rate or at an increased rate relative to the parental strain (17, 18). These data, together with the BPR data presented here (Table 2) make it likely that several *E. coli* ribonucleases can participate in the functional decay of mRNA *in vivo*.

In summary, it appears that when a ribosome binds to the kinase mRNA initiation site *in vitro*, and does not move, it protects the messenger against nuclease attack. Improvements on the cell-free system reported here should allow localization of unique nuclease sensitive sites on any messenger that can be translated *in vitro* and thereby provide further insight into mechanisms of regulation of decay of specific mRNAs.

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