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Transcription-translation and translation-messenger RNA decay coupling: Separate mechanisms for different messengers

(phage T4 infection/deoxynucleotide kinase/α-glucosyltransferase/antibiotics)

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ABSTRACT **Antibiotics were used to inhibit protein synthesis at specific steps in the biosynthetic pathway. In this way, it was possible to study the coupling of protein synthesis to the accumulation of biologically active mRNA in T4-infected *Escherichia coli*. Functional mRNA for the phage enzymes deoxynucleotide kinase (EC 2.7.4.4; ATP:nucleoside-monophosphate phosphotransferase or nucleosidemonophosphate kinase) and α-glucosyltransferase (EC 2.4.1.2; 1,4-α-D-glucan:1,6-α-D-glucan 6-α-glucosyltransferase or dextrin dextranase) accumulated during inhibition of protein synthesis irrespective of the step in the synthesis of protein that was blocked. Under these conditions, however, the rate of mRNA synthesis for both enzymes was significantly inhibited. In contrast, the rate of degradation of these mRNAs was markedly dependent on the step in protein synthesis that was inhibited. That is, the site for mRNase action was different for each message. The most important step in protein synthesis required for the stability of deoxynucleotide kinase mRNA is the initiation step. A single ribosome bound to the 5′ end of the deoxynucleotide kinase mRNA can stabilize the molecule. On the other hand, the initiation event does not seem to be important for stabilizing the α-glucosyltransferase mRNA. Instead, a high ribosome density on the α-glucosyltransferase messenger is required to achieve significant stability. Therefore, in studying messenger metabolism, it is important to focus on the functional stability of specific mRNAs instead of on total messenger since each mRNA can be metabolized differently.**

Little is known about the relationship between transcription and translation and the relationship between translation and messenger decay. However, in *Escherichia coli* the available evidence suggests that in the absence of translation, *trp* mRNA and *lac* mRNA synthesis slow down drastically, and that ribosomes along the length of these molecules protect these mRNAs from rapid decay (1, 2). We are interested in messenger synthesis and decay and have chosen to study phage T4 mRNA metabolism for the following reasons. When T4 infects an *E. coli* cell, host synthesis is shut off and, instead, viral mRNA is exclusively made (3). The availability of *in vitro* RNA directed protein synthesizing systems enables us to study the *in vivo* synthesis and metabolism of specific T4 mRNAs (4, 5). In addition, it is already known that there are at least two classes of mRNA with respect to kinetics of decay (6, 7).

One way to study the interrelationships between protein synthesis and the accumulation of biologically active mRNA is to dissociate the two processes by inhibiting protein synthesis. Since different inhibitors of protein synthesis interfere with translation at different steps, they may be useful in determining those reactions necessary for the accumulation of biologically active mRNA *in vivo*. The questions we are attempting to answer are: What steps, if any, in the translation process are necessary for the synthesis of functional mRNA; and what steps in protein synthesis are necessary for, or protect against, the decay of mRNA?

We have found that while the transcription of two T4 genes, deoxynucleotide kinase (hereafter called kinase) (EC 2.7.4.4; ATP:nucleosidemonophosphate phosphotransferase or nucleosidemonophosphate kinase) and α-glucosyltransferase (α-gt) (EC 2.4.1.2; 1,4-α-D-glucan:1,6-α-D-glucan 6-α-glucosyltransferase or dextrin dextranase), is not strictly coupled to translation, the rate of synthesis of these mRNAs during inhibition of protein synthesis is markedly depressed. Furthermore, our experiments suggest that a ribosome frozen in the initiation site is sufficient to stabilize the kinase mRNA but not the α-gt mRNA. The α-gt messenger is stabilized by ribosomes positioned along the length of the molecule, but the kinase message is not stabilized in this way.

MATERIALS AND METHODS

Bacteria and Phage. The bacterial strains used were *E. coli* B207 and *E. coli* AS19, a mutant of *E. coli* B which is highly permeable to many antibiotics (8). Wild-type phage T4 was used. A multiplicity of infection of 5 was used for strain B207 and 25 for AS19.

Media. Cells were cultured in a glucose-salt medium supplemented with 0.3% casamino acids (9).

Enzyme Synthesis *In Vitro*. This method has been described previously (6, 7, 15). In these assays the amount of enzyme is proportional to the amount of mRNA added.

Enzyme Assays. The assays for kinase and α-gt have been previously described (7, 10). One nmol of [^{14}C]5-hydroxymethyldeoxycytidilic acid phosphorylated equals 1400 cpm; and 1 nmol of glucose transferred from [^{14}C]UDP-glucose to T2 DNA equals 200 cpm.

RNA Extraction. This has been previously described (11).

RESULTS

Action of Antibiotics on Protein Synthesis. We are interested in studying various aspects of mRNA metabolism as they relate to protein synthesis. Many specific inhibitors of protein synthesis exist and their specific sites of action have been well characterized. Consequently, we can experimentally stop protein synthesis at a specific step in the pathway and determine the effect of this on mRNA metabolism *in vivo*. The antibiotics used and their mode and site of action are summarized in Table 1. The table also notes the effects of these drugs on polysome stability. All antibiotics were used at a concentration of 100 μg/ml, which completely inhibits protein synthesis in the bacterial strains used.

Accumulation, Decay, and Rate of Synthesis of T4

Abbreviations: kinase, deoxynucleotide kinase; α-gt, α-glucosyltransferase.

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Table 1. Mechanism of action of antibiotics

Antibiotic	Subunit specificity	Effect on polysomes	Mechanism of action
Chloramphenicol	50 S	Stabilize	Inhibits transpeptidation
Fusidic acid	50 S (EF-G)*	Stabilize	Inhibits aminoacyl-tRNA binding by interaction with EF-G
Puromycin	50 S	Degrade	In place of aminoacyl-tRNA accepts nascent peptide causing a premature release of incomplete peptides
Vernamycin A	50 S	Degrade	Inhibits an early step after initiation
Tetracycline	30 S	Stabilize	Inhibits aminoacyl-tRNA binding to A site

See refs. 26–31.
* EF-G, elongation factor G.

mRNA During Inhibition of Protein Synthesis. It is already known that phage mRNA, as measured by incorporation of [^{14}C]uracil, accumulates in the absence of protein synthesis (12, 13). In addition, Young (6) and Sakiyama and Buchanan (7) have shown that kinase and α-gt mRNAs accumulate when protein synthesis is inhibited by chloramphenicol shortly after infection. Therefore, in our first experiments, the accumulation of kinase and α-gt mRNA in the presence of inhibitors of protein synthesis was determined.

Cells growing at 37° were infected with T4, and, at 4 min after infection, protein synthesis was inhibited. The functional stability, i.e., biological activity of mRNA, was assessed by isolating RNA from phage infected cells at various times and determining the ability of this RNA to direct the synthesis of kinase and α-gt in an *in vitro* protein synthesizing system. The results are given in Fig. 1. Only the data for vernamycin A and puromycin are presented. The results obtained for accumulation of mRNA using tetracycline are essentially the same as those for vernamycin. The other antibiotics give data on accumulation similar to puromycin.

In uninhibited cultures, mRNA for both enzymes accumulates until about 9 min and then declines, so that at about 15 min after infection little functional mRNA is present (Fig. 1 and refs. 6 and 7). However, when protein synthesis is inhibited by addition of vernamycin A 4 min after infection, mRNA for both kinase and α-gt accumulated and is found even at 20–24 min after incubation. Similar results are obtained with puromycin except the duration and magnitude of accumulation are less. The data for the other antibiotics used are presented in Table 2 (columns 1 and 4). These data are presented in the form of rate constants relative to the control (normalized to 1.0). The lower the value, the lower is the rate of accumulation.

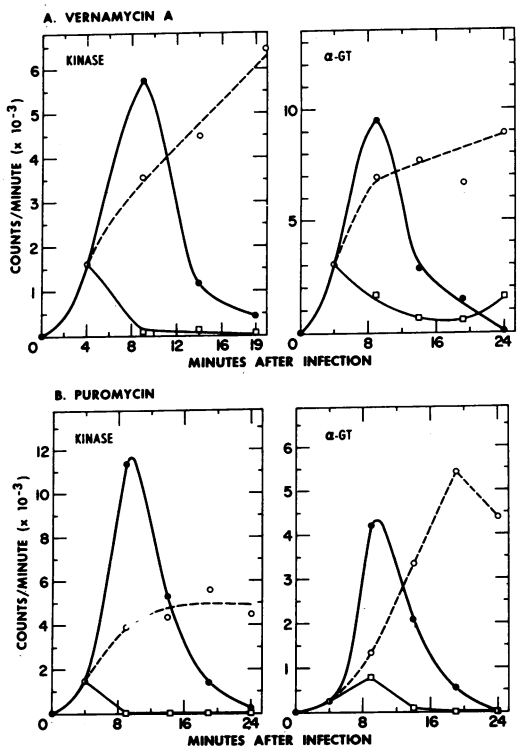

FIG. 1. Accumulation of kinase and α-gt mRNA during vernamycin A or puromycin inhibition of protein synthesis. Growing cells of strain AS19 were infected with T4 and the culture was then divided into three parts. To the first part nothing was added (control) (●); to the second part, (A) vernamycin A or (B) puromycin was added at 4 min (○); and to the third, antibiotic plus rifampicin (300 μg/ml) were added (□). Samples were taken at the indicated intervals; RNA was isolated and used to direct the *in vitro* synthesis of kinase and α-gt. Reaction mixtures incubated without RNA yielded backgrounds of 400 cpm and 900 cpm for kinase and α-gt assays, respectively, which were subtracted from all experimental data. The data for kinase and α-gt mRNA accumulation are not necessarily derived from a single sample of mRNA isolated from one experiment. The experiments were done several times, and representative data for each antibiotic and for each enzyme are presented.

When initiation of messenger synthesis is inhibited by the addition of rifampicin at 4 min after infection, no accumulation of kinase or α-gt mRNA activity is observed. This shows that not all the messengers for these enzymes have been initiated by 4 min after infection but new rounds do occur after the addition of the inhibitor of protein synthesis.

Since the experiment described above is a composite of messenger synthesis and degradation, the results could be due to effects on either of these processes during inhibition of translation or to a combination of both. Consequently, the rate of decay of these messages was determined in experiments similar to those described above, except that new RNA synthesis was inhibited by rifampicin at 9 min. Specifically, 4 min after infection with T4, protein synthesis was inhibited. At 9 min rifampicin was added and RNA was extracted at intervals thereafter and assayed as described above. This experiment measures the stability of mRNA made during inhibition of protein synthesis. The data obtained are given in Table 2 (columns 2 and 5) and are presented in the form of rate constants relative to the control (normalized to 1.0). The lower the value, the more stable is the mRNA, i.e., the greater is the half-life ($t_{1/2}$). The kinetics of decay of this RNA is complex because transcription is

Table 2. Relative rate constants for accumulation, decay, and synthesis of enzyme specific mRNA during inhibition of protein synthesis

Conditions	Relative rate constants for kinase mRNA			Relative rate constants for α-gt mRNA		
	1 Accumulation	2 Decay	3 Synthesis	4 Accumulation	5 Decay	6 Synthesis
Control	1.0	1.0	1.0	1.0	1.0	1.0
Chloramphenicol	0.36	0.71	0.59	0.67	0.24	0.42
Fusidic acid	1.0	0.76	0.86	1.0	0.20	0.44
Puromycin	0.41	1.0	0.74	0.47	0.43	0.45
Vernamycin A	0.63	0.40	0.48	0.64	0.28	0.39
Tetracycline	1.0	0.09	0.38	0.69	0.11	0.30

The rate constants were calculated in the following way, since both accumulation and decay were found to be first order processes, for the first 9 min of infection. The rate of accumulation of mRNA = $dA/dt = kA$. The rate of decay of mRNA = $-dA/dt = k_1A$. The rate of synthesis then = $(k + k_1)A$, where A equals the mRNA content of the cells at any time. Therefore, the rate constant for synthesis = $(k + k_1)$. k, the rate constant for accumulation = $(\log A_8 - \log A_4)/4$, where A_8 = the mRNA content at 8 min and A_4 = the mRNA content at 4 min; and k_1 the rate constant for decay = $(\log 2)/t_{1/2}$, where $t_{1/2}$ = half-life of the mRNA in min. A was calculated from data similar to that presented in Fig. 1 and measures the mRNA content of *in vivo* synthesized RNA in an *in vitro* enzyme synthesizing system. $t_{1/2}$ was calculated from experiments similar to that presented in Fig. 2 except that antibiotic was added at 4 min after infection, and rifampicin 9 min after infection. All data are normalized to the control equal to 1.0. The average actual values for the control rates are: kinase, accumulation, 0.13 min^{-1}, decay, 0.20 min^{-1}, and synthesis, 0.33 min^{-1}; α-gt, accumulation, 0.14 min^{-1}, decay, 0.23 min^{-1}, and synthesis, 0.37 min^{-1}. Several experiments were done in which many points were taken, and these proved to be first order. It was unfortunately impractical to take so many points in each experiment. Several experiments using each drug were performed, and similar rates were observed in each. The rates in the Table are averages of two to four experiments.

occurring in the presence of the antibiotic. Under these conditions we do not know the mode of action of the antibiotics, nor do we know the configuration of the mRNA and its relationship with the membrane and DNA.

Since the rate constants for accumulation and degradation are known for each inhibitor, the rate constant for synthesis can be calculated. This is also shown in Table 2 (columns 3 and 6). The data are again presented relative to the control (normalized to 1.0). The smaller the number, the lower is the rate of synthesis. As can be seen, kinase mRNA is made more rapidly than α-gt mRNA in all cases.

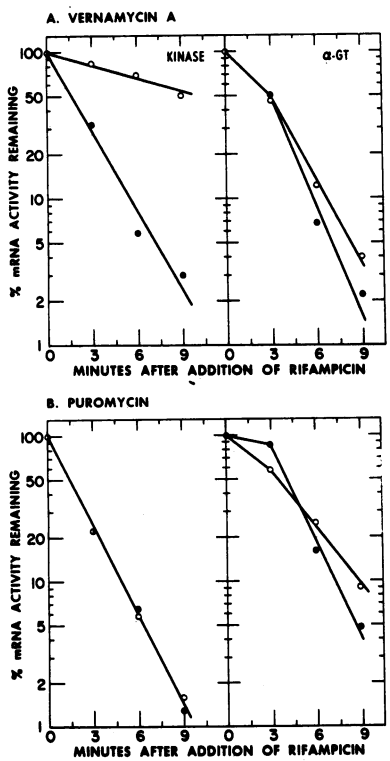

FIG. 2. Decay of kinase and α-gt mRNA in the presence of vernamycin or puromycin. Growing cells of strain AS19 were infected with T4. Nine minutes after infection rifampicin was added to half of the culture (●), and to the other half (A) vernamycin and rifampicin or (B) puromycin and rifampicin were added (○). Samples were taken and the RNA was extracted and analyzed as described in the legend to Fig. 1. In the figure 0 min is the time of addition of the drugs (i.e., 9 min after infection).

Decay of mRNA Made Before Inhibition of Protein Synthesis. The experiments to be described in this section were designed to determine the turnover rate of normal mRNA (i.e., mRNA made before inhibition of protein synthesis) during subsequent inhibition of protein synthesis. Protein synthesis inhibitor and rifampicin were added to cultures simultaneously 9 min after infection. RNA was isolated at intervals thereafter and the ability of this RNA to direct the *in vitro* synthesis of kinase and α-gt was determined. The data obtained using vernamycin A and puromycin are presented in Fig. 2. These data and those obtained using the other antibiotics are given in Table 3 and are presented as $t_{1/2}$, not rate constants, relative to the control (nor-

Table 3. Effect of antibiotics on rate of decay of T4 mRNA synthesized before addition of antibiotic

Conditions	Relative half-life	
	Kinase mRNA	α-gt mRNA
Control (no antibiotic added)	1.0*	1.0
Puromycin	1.0†	1.4
Chloramphenicol	1.4	*4.6*
Fusidic acid	2.1	*5.0*
Vernamycin A	*5.1*	2.0
Tetracycline	*4.7*	*9.0*

* The half-life of the control was normalized to 1.0. The actual half-life is 1.2–1.8 min, depending on the experiment.

† All half-lives are normalized to the control, which was determined simultaneously in every experiment. The data are averages of at least two separate experiments and are taken from data as given in Fig. 2. Significant stabilization is indicated by the *italics*.

malized to 1.0). The higher the number, the larger is the $t_{1/2}$ and therefore the more stable is the mRNA. It is important to note that the different antibiotics stabilize each mRNA differently. The results will be discussed in the *Discussion*.

RNA isolated 9 min after infection (before addition of drugs) was also analyzed for size on sucrose density gradients. The data presented elsewhere (14, 15) show that the messengers for both kinase and α-gt are homodisperse and the same size (14.5 S) at this time after infection. Therefore, the differences observed between the stability of the two messengers in the presence of the different antibiotics do not relate to the size differences between kinase and α-gt mRNAs. Furthermore, we know that the majority (if not all) of the mRNA is associated with polysomes (15) at this time after infection.

DISCUSSION

Stability of mRNA reflects the availability of sites for inactivation (i.e., for nuclease action). So it follows that if the sites are not available, mRNA is stable, and vice versa. For example, if a ribosome covers a unique site involved in mRNA decay (a ribosome can cover about 40 nucleotides; see refs. 16 and 17), the mRNA would be stable. Alternatively, ribosomes bound to a portion of (or the entire) mRNA molecule may melt a double-stranded configuration which the mRNA can assume. This denaturation can then either make a site available for RNase (to a single-stranded mRNase type) or, alternatively, can make the site unavailable (to an RNase III type, which specifically degrades double-stranded RNA). We can interpret all our data by interactions between ribosomes and mRNA, which can result in stabilization or labilization of mRNA.

It is already known that there are at least two classes of phage mRNA with respect to their kinetics of decay (6, 7). That is, kinase mRNA that accumulates *in vivo* in the presence of chloramphenicol is unstable, whereas α-gt mRNA accumulated under the same conditions is stable. This observation proved useful to us as an aid in exploring the possible specificity of mRNases. The importance of the present investigation relates to the use of antibiotics as tools in studying mRNA degradation. The fact that it is possible to attain a specific mRNA-ribosome interaction *in vivo* by use of antibiotics is an interesting finding. It has also enabled us to study the role of ribosome-mRNA interaction in mRNA metabolism.

The most significant finding we present is that the *in vivo* decay of T4 mRNA present in polysomes is dependent on the step in protein synthesis that is inhibited, and consequently, on the interaction of ribosomes with mRNA. This conclusion is reached by interpretation of the data presented in Fig. 2 and Table 3. In these experiments antibiotic was added to cells 9 min after normal infection. It is known that essentially all this mRNA is bound to polysomes (15) and that the size of both kinase and α-gt messengers is the same and homodisperse at this time (14, 15). The most important step required for the stability of kinase mRNA is the initiation step (or an early event in protein synthesis). This conclusion is reached from the following data (Table 3). Vernamycin A, which inhibits an early step at or after the initiation event and freezes a ribosome in that configuration, and tetracycline, which allows initiation but then freezes the ribosome in that configuration, both stabilize kinase mRNA. (See Table 1 for action of antibiotics.) In contrast, puromycin allows normal initiation and does not affect normal decay of the kinase mRNA. Chloramphenicol slows down the rate of peptide bond formation but does not affect initiation or completely inhibit ribosome movement and only slightly decreases the rate of kinase mRNA decay. Fusidic acid allows at least one or two translocations and possibly more *in vivo* following the initiation step and only marginally decreases the rate of kinase mRNA decay. We argue that if the initiation site on the mRNA (or a site close to it) is covered by a ribosome, then kinase mRNA is stable, perhaps because the primary available inactivation site (mRNase site?) is at that region. Others (18, 19) have similarly concluded from different evidence that the 5′ end of the mRNA has a special role in stabilizing bacterial mRNA.

On the other hand, the initiation site does not seem to be the important site for α-gt degradation. Vernamycin A, in contrast to the results obtained with kinase mRNA, marginally stabilizes α-gt mRNA. All methods that result in some preservation of the polysome configuration (chloramphenicol, fusidic acid, and tetracycline) stabilize the α-gt mRNA, whereas those which result in polysome decay or run-off do not (puromycin and vernamycin A). It therefore seems that it is necessary to have a high ribosome density on the α-gt mRNA to get significant stability. This finding is consistent with the observation that functional α-gt mRNA can be isolated *in vivo* only on polysomes containing at least three to six ribosomes, whereas functional kinase mRNA has been isolated containing as few as one ribosome (15). It is interesting to note that the decay pattern of normal 9 min α-gt mRNA is biphasic. That is, for 3 min, α-gt mRNA is far more stable than for the succeeding 6 min (Fig. 2). In line with our hypothesis, it may be that ribosomes initiate protein synthesis on an α-gt mRNA molecule for about 3 min *in vivo*, following which the rate of ribosome run-off exceeds the rate of ribosome initiation. This mechanism could leave the α-gt mRNA without the ribosome density necessary for protection against mRNase action.

We also show that α-gt mRNA is more stable than kinase mRNA irrespective of the step in protein synthesis that is inhibited (Table 2, columns 2 and 5). At present, we do not understand why this is so, but it is possible that the effect is related to the fact that kinase mRNA can be synthesized as a monocistronic messenger (5, 20, 21) whereas α-gt mRNA appears to be synthesized at the promotor distal end of a polycistronic messenger (22, 23). Of special interest is the determination of why tetracycline causes the extreme stability of both the kinase mRNA and α-gt mRNA made in the presence of this antibiotic (Table 2).

The fact that mRNA can be synthesized for a long period of time subsequent to the addition of antibiotic indicates, in agreement with previous work (7), that a phage induced protein is required for termination of early mRNA synthesis.

Finally, we show that functional T4 mRNA is made during inhibition of protein synthesis irrespective of the step in the synthesis of protein that is blocked (Table 2, columns 3 and 6). Furthermore, it was shown that kinase, a monocistronic messenger, is made more rapidly than α-gt, a polycistronic mRNA. This may be due to a polarity effect on the α-gt mRNA, which occupies the distal portion of the polycistronic messenger. It is not clear to us why the antibiotics should affect the synthesis of mRNA, but it is known that antibiotics can inhibit the synthesis of certain bacterial messengers (24, 25). We therefore conclude that T4 mRNA synthesis is not strictly coupled to translation. However, the maximal rate of mRNA transcription obviously takes place in the presence of its translation.

It should be noted that all the experiments reported in this

investigation were done on the effect of the drugs on intact cells. The system can just as easily be adapted for *in vitro* analysis.

Our work is important because it shows that one must study specific mRNAs since each messenger may be metabolized differently. Also, it opens a new approach to the study of mRNA metabolism and points to the importance of specific ribosome-mRNA interactions in mRNA decay. It is also important to note that we are measuring functional inactivation rather than chemical degradation. Since the mRNA can be chemically stable but functionally inactive, if, for example, there is a single nick in the mRNA, the loss of biological activity is the important parameter to study.

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