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EFFECT OF RIBOSOMAL LOADING ON THE STRUCTURAL STABILITY OF BACTERIOPHAGE T7 EARLY MESSENGER RNAs

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SUMMARY. The in vivo structural stabilities of the T7 early mRNAs were measured and found to vary according to whether chloramphenicol or puromycin were added before or after infection with phage T7. These antibiotics had little effect upon messenger stability when they were added prior to infection. When chloramphenicol (but not puromycin) was added after completion of T7 early mRNA synthesis, the structural stability of the messages was enhanced. Messages which are inefficiently translated in vivo due to altered 5'-termini were not stabilized by the late addition of chloramphenicol. We interpret these results to mean that ribosomal protection of the T7 early mRNAs is responsible for the increase in messenger structural stability in the presence of chloramphenicol.

INTRODUCTION

As a result of the report by Summers (1) that messenger RNA of bacteriophage T7 accumulates as acid precipitable material in T7-infected E. coli, the stability of T7 mRNA has received considerable attention. Marrs and Yanofsky (2) demonstrated that this stability is T7-specific, since the mRNA of the trp operon is degraded at a normal and rapid rate in T7-infected cells. While these early experiments measured the fate of T7 mRNA in terms of its acid precipitability and hybridizability to T7 DNA, more recent work in which the physical integrity of the messenger RNA was monitored by polyacrylamide gel electrophoresis indicates that T7 mRNA is structurally unstable, having a half-life of 1.8 to 6.3 minutes, depending upon the message examined (3). Yamada et al., (4 & 5) have shown that the T7 early mRNAs are functionally unstable, and have attributed this instability to a loss of the structural integrity of the messengers.

By translating messenger RNAs from T7-infected cells in an in vitro system, Schiecher and Bautz (6) determined that the messenger RNA of gene 1

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has a functional half-life in infected cells of only 2.5 minutes, and that the rate of inactivation of the message is not affected by the presence of an amber codon within the gene. The latter observation is in contrast with the view that in E. coli, an amber codon within a transcript results in destabilization of the message, particularly that portion which is transcribed from regions of the template that are distal to the mutation. Since amber (nonsense) codons are known to prevent the passage of ribosomes along the message, the observed instability of messages containing such codons has been interpreted to mean that mRNA molecules with bound ribosomes are shielded from nucleolytic attack. In view of the observations by Schliecher and Bautz, the question arises as to whether T7 mRNA is different from host mRNA in that it is not protected by ribosomes.

In this report, we have examined the effect of ribosome binding on the stability of T7 mRNA in two ways: (1) by examining the effect of antibiotics which prevent or enhance the association of ribosomes with messenger RNA, (2) through the use of phage mutants in which the structure of certain mRNAs have been altered so that ribosomal binding is decreased.

MATERIALS AND METHODS.

Bacteriophage and Bacteria. All strains of bacteriophage T7 were grown and purified as described by Studier (7, 8). E. coli B was obtained from the laboratory of Dr. E.K.F. Bautz.

Chemicals and Radiochemicals. Sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylene-bis-acrylamide (BIS), N,N,N',N',-tetramethylethylenediamine and ammonium persulfate were purchased from Biorad (Richmond, California). Puromycin was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio); chloramphenicol from Calbiochem, (San Diego, California); and rifampin from Schwarz-Mann (Orangeburg, New York). Radioactive phosphate was obtained as orthophosphate from ICN Pharmaceuticals (Irvine, California).

Pulse labeling of T7 mRNA. E. coli B, grown at 30°C in B2 medium (9) to a density of 6-8 x 10^8 cells/ml, were UV irradiated (8) and infected at a multiplicity of 10 phage particles/cell. Labeled phosphate (20μCi/ml) was added immediately after infection. Incorporation of label into RNA was blocked six minutes after infection by the simultaneous addition of rifampin (.40mg/ml) and unlabeled phosphate (0.02M). Aliquots of 100μl were withdrawn at intervals thereafter and mixed with 1 ml of ice cold M9 medium (8). The cells were harvested by centrifugation and lysed by exposure to 0.1% SDS in a boiling water bath (8).

Electrophoresis of RNA. Samples from infected cells were analyzed by electrophoresis on gradient gels of 2.2 to 10% acrylamide in 0.1M PO_4 buffer, pH 7.0, as described by Studier (8). The gels were dried and exposed to Kodak RP-Royal X-Omat film. Densitometer tracings of the film were made with a Joyce-Loebl scanning microdensitometer.

RESULTS

Structural stability of early T7 mRNAs. Transcription of the late genes of bacteriophage T7 requires the synthesis of a phage-specified RNA polymerase, the product of gene 1 (10); in cells infected with phage defective in gene 1, only the early T7 mRNA species are produced. To determine the structural stability of the early T7 mRNAs, the early RNAs were labeled with radioactive phosphate ($^{32}PO_4$). Six minutes after infection, the incorporation of label into nascent RNA was blocked by the simultaneous addition of excess unlabeled PO_4 and rifampin [an inhibitor of the bacterial RNA polymerase (10)]. At defined intervals thereafter, samples of the culture were harvested, rapidly lysed in the presence of SDS, and analyzed by electrophoresis in polyacrylamide gels (Figure 1). The amount of labeled RNA remaining in each band at intervals after the cessation of RNA synthesis was determined by desitometric analysis of the film. By plotting the logarithm of the density of each band as a function of the time after infection (Figure 3) we have determined the structural half-life of each mRNA under a variety of conditions (Table 1). As a general feature, we observe that individual messenger stability is inversely related to the molecular weight of the RNA species.

Effect of Antibiotics. Two drugs which are known to affect the association of ribosomes with bacterial mRNA are chloramphenicol and puromycin. Whereas puromycin leads to the dissociation of bound ribosomes from mRNA with the resultant breakdown of polysomes, chloramphenicol inhibits the translocation of ribosomes and prevents their release from the message, thus leading to stabilization of existing polysomes (for review, see 11). On the other hand, chloramphenicol inhibits the association of free ribosomes with nascent mRNA and prevents the incorporation of newly synthesized mRNA into polysomes (ibid).

With the exception of gene 1 mRNA, which was slightly destabilized in

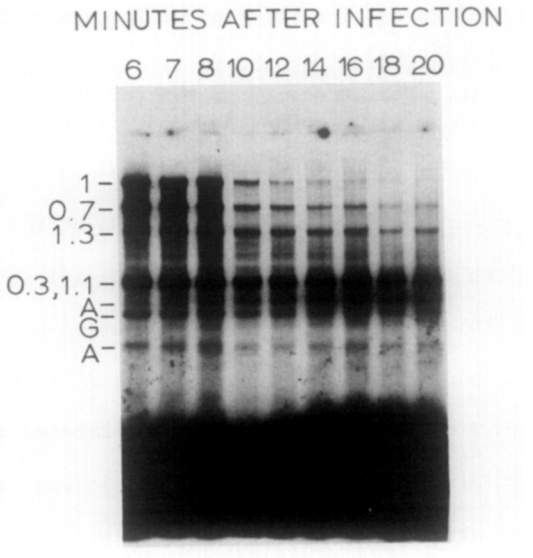

Fig. 1. Structural decay of T7 early mRNAs.

E. coli B, grown in low phosphate media, were UV irradiated and infected with T7 am 193,27. Immediately after infection ^{32}P-[PO_4] was added to 20μCi/ml. At 6 minutes post-infection, incorporation of label into viral RNA was terminated by the addition of rifampin (400μg/ml) and competitor PO_4 (0.2M). Samples were withdrawn at the times indicated above the figure and prepared for electrophoresis as described in Materials and Methods. Electrophoresis was for 4 hours at 70 volts followed by 12 hours at 15 volts on gradient (2.2 - 10%) gels of polyacrylamide. In this particular experiment, chloramphenicol was added to 200μg/ml 2 minutes prior to infection. Following electrophoresis, the gel was dried and exposed to x-ray film. The T7 early RNAs are identified by the appropriate gene number to the left of the autoradiogram. In addition, the position of three short initiation fragments of viral mRNA produced during processing (13) are identified by A,G,A (see Fig. 2). Note that the mRNAs of genes 0.3 and 1.1 are not resolved in these gels.

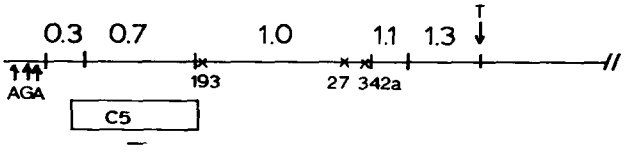

Fig. 2. Relative positions of the T7 early genes

Physical map of the T7 early region indicating the relative positions of the deletions and point mutations used in this study (7). Numbers above the line indicate the T7 early genes. Three promotors for E. coli RNA polymerase are located near the left end of the genome which permit initiation with either ATP or GTP as indicated. The early messengers are generated by transcription from the above promotors to a termination site (T) located to the right of gene 1.3, and subsequent RNAase III cleavage of the polycistronic message. The cleavage sites are designated by vertical bars separating the early genes (13). [ ], deletion; X, amber codon.

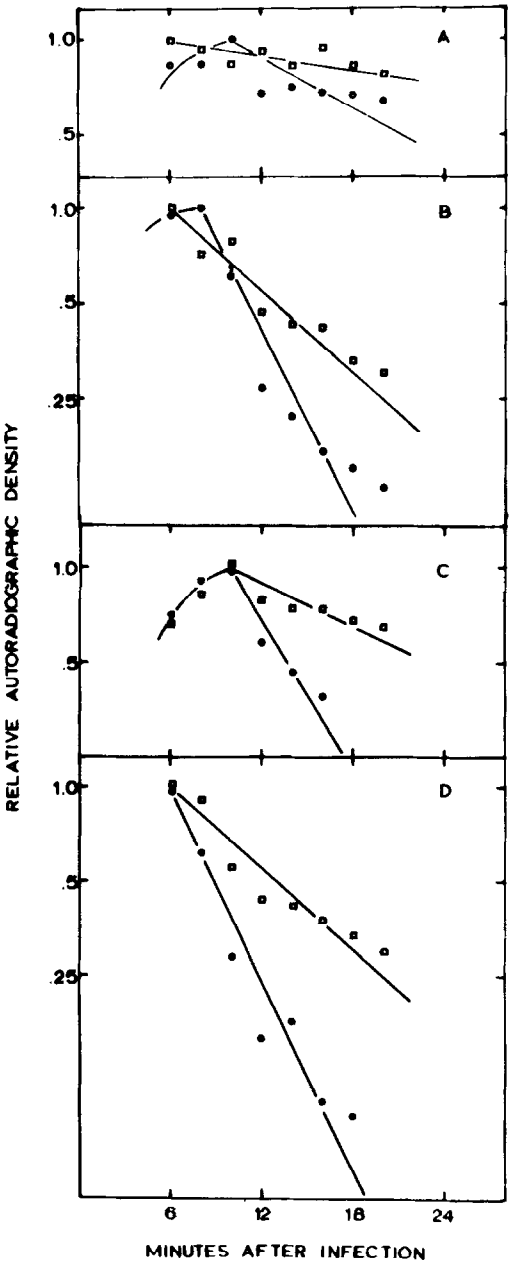

Fig. 3. Structural decay rates of T7 early mRNAs.

Autoradiograms such as that shown in Figure 1 were scanned with a Joyce-Loebl microdensitometer. The densities obtained for each of the messages were normalized to 1.0 (the highest density attained by a message during the experiment). Points are plotted as relative autoradiographic density vs. time after infection. The slopes obtained from the linear portions of these and similar curves were used to determine the half-lives of the messages: A, Gene 0.3,1.1 mRNA; B, Gene 0.7 mRNA; C, Gene 1.3 mRNA; D, Gene 1.0 (am 342a) mRNA. - O - O -, chloramphenicol added prior to infection; - □ - □ -, chloramphenicol added at six minutes after infection.

Table 1. Structural Decay Rates of T7 Early mRNAs[a]

Messenger[b]	Antibiotic Treatment[c]				
	N	CME	CML	PME	PML
0.3,1.1	9.0	10.2	36.0	-	12.0
1.3	5.5	4.3	12.5	4.5	5.3
0.7	3.6	3.0	7.0	2.5	4.2
1 (*am*193,27)	2.2	2.0	3.8	2.0	-
1 (*am*342a)	2.3	2.3	6.5	-	3.2
1 (WT)	-	1.8	-	-	-
1 (C5,*am*342a)	2.8	2.9	2.8	-	-

a) Half-lives of messengers were determined from plots as depicted in Figure 3 and are given in minutes.
b) T7 messengers are identified by gene number. The information in parentheses indicates mutations which affect their structure (see Figure 2). WT = wild-type.
c) N = No antibiotic added, CME = chloramphenicol added to 200 μg/ml prior to infection, CML = chloramphenicol added to 200 μg/ml at six minutes post-infection, PME = puromycin added to 300 μg/ml prior to infection, PML = puromycin added to 300 μg/ml at six minutes post-infection.

the presence of the drugs, the addition of chloramphenicol and puromycin to bacterial cultures prior to infection did not markedly affect the stability of most T7 early mRNAs (Table 1). In contrast, when chloramphenicol (but not puromycin) was added to cultures at six minutes after infection, a marked increase in the stability of all T7 mRNAs was observed (Figure 3 and Table 1).

Effect of amber mutations. The effect of an *amber* codon upon the stability of the gene 1 message was analyzed in several experiments. Whereas chloramphenicol stabilized the gene 1 message when this drug was added to infected cultures after the synthesis of T7 early mRNA, the presence of an *amber* codon near the 5'-terminus of the message diminishes the protective effects of this drug (Table 1). Since messages having *amber* codons near their 3'-termini are stabilized by chloramphenicol (Figure 3, Panel D), we interpret these results to mean that the protective effect of chloramphenicol on T7 mRNA stability requires the presence of ribosomes along the message.

To further test this hypothesis, we have determined the effect of chloramphenicol on the stability of mRNA of T7 (C5, 1-342a). This T7 mutant

has a deletion (C5) which eliminates all of gene 0.7, and results in the fusion of the gene 0.3 mRNA with that of gene 1 (Figure 2). The gene 1 portion of the resultant fusion transcript is poorly translated (8); consequently, one might expect that this message would have a relatively low concentration of ribosomes and should not be stabilized by the late addition of chloramphenicol. As predicted, no stabilization was observed (Table 1).

DISCUSSION.

In this report, we have presented evidence that chloramphenicol stabilizes T7 mRNA and that this effect is dependent upon the presence of bound ribosomes along the message. Previous investigators have reported that chloramphenicol, when added prior to infection, does not affect messenger stability (3). This observation is consistent with the finding that chloramphenicol prevents the association of free ribosomes with nascent mRNA, and with our findings that the stabilizing effect of this drug is dependent upon the presence of bound ribosomes on the mRNA.

In view of the observation that T7 mRNA, like host mRNA may be stabilized by bound ribosomes, it is perhaps surprising that the addition of puromycin (or of chloramphenicol prior to infection) does not destabilize the T7 mRNA. One possibility to account for this finding is that degradation of the T7 early mRNAs is ordinarily sufficiently slow so that the rate of nucleolytic attack is not affected by the transient (and relatively rapid) passage of ribosomes over the mRNA. In contrast, if ribosomal movement is slowed down or blocked, these mRNAs are afforded appreciable protection.

Alternatively, any decrease in stability caused by these drugs may be difficult to detect with the techniques employed in these experiments. We have observed that the gene 1 mRNA (the most unstable of all T7 early mRNAs) is slightly destabilized by puromycin or by chloramphenicol added prior to infection.

The mechanism by which T7 (or host) mRNA is degraded is unclear. Since the addition of chloramphenicol or puromycin prior to mRNA synthesis does not markedly affect the stability of the T7 early mRNAs, we conclude that ribosomal binding is not required for degradation of these messengers.

While this manuscript was in preparation, Cohen et al., (12) reported

the use of inhibitors of protein synthesis in analyzing the mechanisms by which the mRNAs of bacteriophage T4 are degraded. In agreement with our findings, T4 mRNAs may also be stabilized by bound ribosomes under appropriate conditions.

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