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Translation of T7 RNA In Vitro Without Cleavage by RNase III

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T7 early mRNA's are generated from a high-molecular-weight precursor RNA by site-specific RNase III cleavage. When T7 DNA is transcribed in vitro by *Escherichia coli* RNA polymerase, the transcript is a large, single-piece RNA equivalent to the in vivo precursor RNA. The T7 RNA synthesized in vitro can be translated as a polycistronic messenger without cleavage by RNase III. All T7 early proteins are synthesized in an RNase III-free, protein-synthesizing system directed by the uncleaved T7 RNA.

The early region of the bacteriophage T7 genome, which occupies the leftmost 20% of the T7 DNA, is transcribed by the host *Escherichia coli* RNA polymerase and generates at least five monocistronic T7 early mRNA's corresponding to T7 early genes 0.3, 0.7, 1.0, 1.1, and 1.3 (11, 17, 18). However, when T7 DNA is transcribed in vitro by purified *E. coli* RNA polymerase, the transcripts are mostly high-molecular-weight, single-piece RNA molecules corresponding to the entire early region of T7 DNA (3, 12, 13). Dunn and Studier (4, 5) showed (i) that in vivo the individual T7 early mRNA species are the cleavage products of a large, single-piece precursor RNA (molecular weight, ca. 2.2×10^6), (ii) that the cleavage is catalyzed by host enzyme RNase III, which is specific for double-stranded RNA (15), and (iii) that this precursor RNA can accumulate in RNase III$^-$ mutant cells infected with T7 phage (4, 14) and is similar in size to the T7 RNA synthesized in vitro by *E. coli* RNA polymerase. The T7 RNA synthesized in vitro generates several smaller pieces of RNA species after RNase III treatment that are identical to the T7 early mRNA's produced in vivo with respect to both size (3) and the 5'- and 3'-terminal base sequences (10, 16).

From these observations, it appeared likely that the processing of the large precursor RNA by RNase III to generate several monocistronic T7 early mRNA's is a necessary step to facilitate translation of T7 early mRNA's. The fact that T7 early mRNA's contain an extensive secondary structure (21) further implies that without the processing by RNase III the protein synthesis initiation signals of the RNA are masked for translational initiation. In fact, Hercules et al. (6) reported that the precursor RNA that accumulates in T7-infected RNase III$^-$ cells is a poor messenger to direct the synthesis of T7 proteins in vitro unless treated with RNase III.

Our study, however, showed that the in vitro transcript from T7 DNA, a large-molecular-weight, single-piece RNA, can be translated in vitro as a polycistronic mRNA to produce all T7 early proteins without cleavage by RNase III.

In this study, T7 early RNA was synthesized in vitro by purified *E. coli* RNA polymerase on purified T7 DNA as described previously (8) in the absence or presence of RNase III. RNase III was prepared from *E. coli* cell extracts by the method of Crouch (2), resulting in an 800-fold purification. In the absence of RNase III, the RNA synthesized in vitro was mainly large, single-piece RNA species with molecular weights of about 2.2×10^6 to 2.5×10^6 (3, 12, 13) (Fig. 1A). In the presence of RNase III, several pieces of RNA corresponding in size to the T7 early mRNA's (11, 18) were produced (Fig. 1B).

The large, single-piece T7 RNA (Fig. 1A) formed polysomes in an f-Met-tRNA$_f$ binding reaction with ribosomes from which RNase III, but not the initiation factors, had been removed by being washed three times with 0.2 M KCl (Table 1). The result indicates that the large-size T7 RNA can act as a single-piece polycistronic messenger with several initiation codon regions accessible to the ribosomes (Fig. 2A). On the other hand, RNase III-treated RNA, which had generated several pieces of RNA species (Fig. 1B), formed f-Met-tRNA containing initiation complexes composed mainly of single ribosomes (Fig. 2B), indicating that each T7 early mRNA species has only one initiation site for protein synthesis.

From a similar f-Met-tRNA binding reaction, which employed [^{3}H]uridine-labeled T7 RNA synthesized in vitro, ^{3}H-labeled T7 RNA was

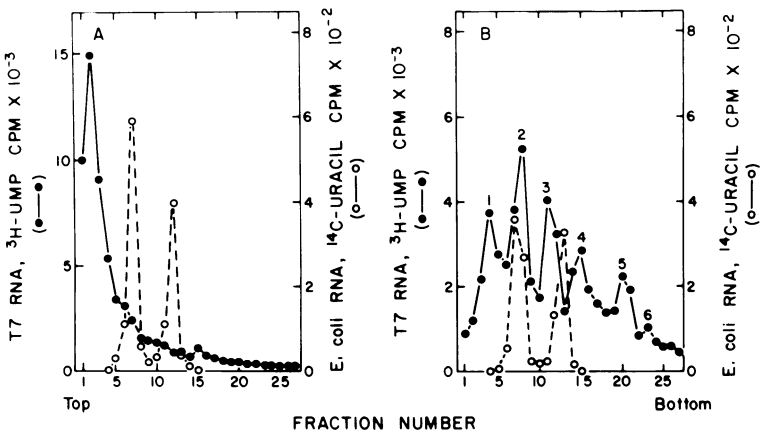

FIG. 1. *Acrylamide-agarose gel electrophoretic patterns of T7 RNA synthesized in vitro (A) and RNA cleaved by RNase III (B). T7 RNA was synthesized in vitro at 37 C for 45 min in a 0.25-ml reaction mixture containing: T7 DNA, 4.8 μg; purified E. coli RNA polymerase (8), 3 μg; Tris-hydrochloride (pH 7.9), 0.04 M; $MgCl_2$, 0.01 M; KCl, 0.1 M; mercaptoethanol, 0.012 M; ATP, CTP, GTP, and UTP, each at 0.2 mM; [^{3}H]UTP (20 Ci/mmol), 2 μCi. To generate cleaved T7 RNA, a parallel reaction was carried out in the presence of 1.8 μg of RNase III (about 800-fold purified, 53 U/μg). RNA was extracted and analyzed by electrophoresis in a 2.75% acrylamide-0.5% agarose disc-gel with [^{14}C]uracil-labeled marker 23S and 16S E. coli ribosomal RNAs (20). Gels were sliced, and the radioactivity of gel slices was determined as described previously (13). Electrophoresis was from left to right in the figure. (A) T7 RNA synthesized in vitro. (B) T7 RNA synthesized in vitro, cleaved by RNase III. Symbols: ●, T7 RNA; ○, marker rRNA. The materials that migrated in peaks 1 through 6 of (B) correspond to: 1, unidentified; 2, gene 1 mRNA; 3, gene 0.7 mRNA; 4, gene 1.3 mRNA; 5, a mixture of gene 0.3 mRNA and gene 1.1 mRNA; 6, unidentified (3, 11-13, 18).*

reextracted and analyzed by gel electrophoresis. Most of the T7 RNA added to the reaction (Fig. 3A) remained intact after the formation of initiation complexes (Fig. 3B). This result supports the notion that the large, single-piece RNA acts as a polycistronic messenger with several initiation sites for translation.

In an in vitro protein-synthesizing system that contained RNase III (composed of crude ribosomes containing RNase III and an S_{200} fraction free from RNase III [Table 1]), the T7 RNAs synthesized in vitro without RNase III cleavage (Fig. 1A) and with RNase III cleavage (Fig. 1B) were equally active in directing the synthesis of all T7 early proteins. Figure 4A and B shows the gel electrophoretic patterns of the proteins synthesized. As shown previously (17, 18), using T7 mutant phages with mutations in the early genes, the proteins synthesized in the in vitro system were identified as T7-specific RNA polymerase (gene 1 product, peak 1 in Fig. 4A), a mixture of T7 protein kinase (gene 0.7 product) and T7 DNA ligase (gene 1.3 product, appearing as peak 2 in Fig. 4A), and a mixture of gene 0.3 and gene 1.1 products (peak 3 in Fig. 4A).

An important result was obtained with another in vitro protein-synthesizing system composed of RNase III-free ribosomes and RNase III-free S_{200} fraction. In this RNase III-free system, the uncleaved, large T7 RNA was active as messenger to produce all T7 early proteins (Fig. 4C), and the amount of T7 early proteins produced in this system was about the same as that produced in the presence of RNase III-cleaved T7 RNA (Fig. 4D). We have confirmed that the proteins synthesized in vitro were actually active enzymes by assaying the activities of T7-specific RNA polymerase (Table 2) and T7 DNA ligase.

However, the large, [^{3}H]uridine-labeled T7 RNA, reextracted from the in vitro system after protein synthesis had occurred, was partially degraded. In the gel electrophoretic analysis, the RNA showed a broad size distribution without any discrete peaks corresponding to RNase III-cleaved products. We assume that a trace amount of RNase III activity in the S_{200} fraction was not responsible for the partial degradation of the T7 RNA, because an incubation of the same T7 RNA in an RNase III-free mixture (all components of the protein-synthesizing system except S_{200}) in the presence of 1.5 U of purified RNase III did not cause any degradation of the RNA. Since the S_{200} fraction used in the protein-synthesizing system contained less than 0.7 U of RNase III activity (Table 1) but a detectable amount of nonspecific RNase activity [assayed by solubilization of ^{14}C-labeled poly(U)], the observed partial degradation of the

TABLE 1. *RNase III activity in subcellular fractions of E. coli*[a]

Subcellular fraction	RNase III	
	Total activity units	% Distribution
S_{30} (crude extract)	149×10^3	100
Crude ribosomes	89.0×10^3	60
0.2 M KCl-washed material from crude ribosomes (first wash)	80.5×10^3	54
Ribosomes washed three times with 0.2 M KCl	0	0
S_{200} (high-speed supernatant fraction of S_{30})	77.5×10^3	52
S_{200} material retained by DEAE 52[b]	$<10^3$	<0.6

[a] *E. coli* D10 F⁻(RNase I⁻) was grown in M9-Casamino Acids-glucose medium, and the S_{30} fraction, crude ribosomes, and S_{200} fraction were prepared, as described previously (19), with TMA buffer (0.01 M Tris-hydrochloride [pH 7.9], 0.01 M $MgCl_2$, 0.022 M NH_4Cl, 1 mM dithiothreitol and 5% glycerol). Crude ribosomes (10 to 15 mg/ml) were washed with 0.2 M KCl in TMA buffer overnight and pelleted by centrifugation to remove the KCl solution. This washing process was carried out three times to yield RNase III-free ribosomes. The S_{200} fraction was absorbed to a DEAE 52 column equilibrated with TMA buffer. The column was washed with TMA buffer and then eluted with TMA buffer containing additional 0.25 M NH_4Cl. The column did not absorb any RNase III activity (15). RNase III activity was assayed by measuring the loss of radioactivity from the double-stranded RNA substrate ^{3}H-labeled poly[r(A-U)] (2, 15). The assay mixture contained in 0.1 ml: 0.02 M Tris-hydrochloride (pH 7.9), 0.01 M magnesium acetate, 0.1 M NH_4Cl, 1 mM mercaptoethanol, 5% glycerol, and 10^4 counts/min of ^{3}H-labeled poly[r(A-U)] (4.1×10^3 counts/min per nmol of nucleotide). After an incubation at 37 C for 10 min, trichloroacetic acid-precipitable radioactivity was measured. One activity unit was defined as the amount of RNase III that converted 1 nmol of ^{3}H-labeled substrate to acid-soluble material per hour under the conditions used. ^{3}H-labeled poly[r(A-U)] was synthesized from [^{3}H]ATP and [^{3}H]UTP by using purified *E. coli* RNA polymerase and poly[d(A-T)] as described by Crouch (2).

[b] S_{200} material retained by DEAE 52 contained about 0.7 U of RNase III activity in the amount (20 μl and 100 μg of protein) that was added to the protein-synthesizing system.

T7 RNA is very likely due to the nonspecific RNase in the S_{200} fraction. Specific and complete cleavage of the large T7 RNA appears to require a large amount of RNase III (50 to 100 U, see legend of Fig. 1).

From the results presented here, we suggest that the processing of the high-molecular-weight precursor T7 RNA by RNase III is not

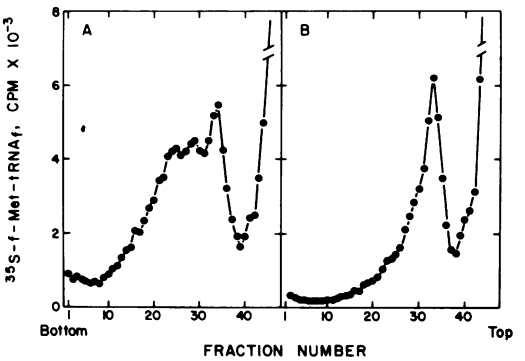

FIG. 2. *Sucrose gradient patterns of protein synthesis initiation complexes formed in the presence of T7 RNA synthesized in vitro (A) and RNA cleaved by RNase III (B). Uncleaved and RNase III-cleaved T7 RNAs were synthesized as described in the legend of Fig. 1, except that [^{3}H]UTP was omitted from the reaction mixture. The formation of initiation complexes was carried out at 35 C for 15 min in the following mixture in a total volume of 0.1 ml: Tris-hydrochloride, (pH 7.2), 0.05 M; NH_4 Cl, 0.05 M; magnesium acetate, 5 mM; GTP, 0.5 mM; mercaptoethanol, 5 mM; ribosomes (RNase III free, washed with 0.2 M KCl three times; Table 1), 420 μg; ^{35}S-labeled f-Met-tRNA$_f$, 5 × 10^5 counts/min; and uncleaved T7 RNA (15 μg) (A) or RNase III-cleaved T7 RNA (22.5 μg) (B). The reaction mixture was fixed at 0 C for 5 min by the addition of 0.4 ml of buffer containing 0.05 M Tris-hydrochloride (pH 7.9), 0.05 M KCl, 0.01 M magnesium acetate, and 1.25% (wt/vol) glutaraldehyde (9). Fixed mixture was placed on a 14 to 28% sucrose gradient containing 0.05 M Tris-hydrochloride (pH 7.9), 0.05 M KCl, and 0.01 M magnesium acetate and centrifuged at 40,000 rpm for 80 min in an SW41 rotor. The radioactivity of each gradient fraction was counted. Centrifugation was from the right to left in the figure. (A) Initiation complexes formed with uncleaved T7 RNA. (B) Initiation complexes formed with RNase III-cleaved T7 RNA.*

mandatory for the synthesis of T7 early proteins at least in vitro, although the cleavage of the RNA by RNase III is specific and generates functionally active T7 mRNA species. The large, single-piece T7 RNA synthesized in vitro can be used to initiate protein synthesis as a polycistronic messenger without cleavage by RNase III, and all T7 early proteins are produced. Our results are not in accord with those of Hercules et al. (6), who reported that the precursor RNA accumulated in T7-infected RNase III⁻ cells (a male strain, AB105, which is not a natural host of T7) required RNase III cleavage to become an active messenger in vitro to direct the synthesis of T7 proteins.

On the other hand, our results are supported by the recent finding by Dunn and Studier (5) that an F⁻ strain of *E. coli* (natural host of T7)

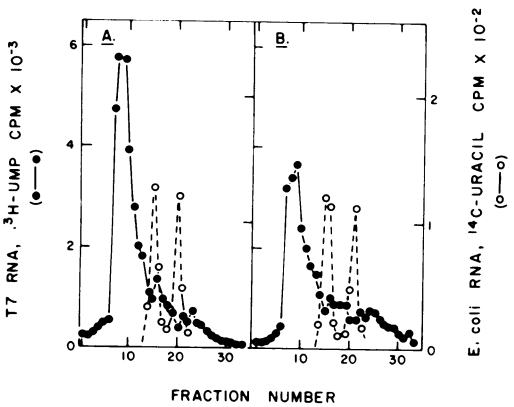

FIG. 3. *Acrylamide-agarose gel electrophoretic patterns of in vitro synthesized T7 RNA before (A) and after the initiation complex formation (B). T7 RNA was synthesized in vitro in the absence of RNase III as described in the legend of Fig. 1. The formation of the translational initiation complex was carried out as described in the legend of Fig. 2, using RNase III-free ribosomes, except that 9 × 10^4 counts/min per 19 μg of ^{3}H-labeled T7 RNA and non-radioactive f-Met-tRNA$_f$ were present in the reaction mixture. RNA was extracted from the reaction mixture before (A) and after (B) incubation for 15 min at 37 C and analyzed by electrophoresis as described in the legend of Fig. 1. (A) T7 RNA before the initiation complex formation. (B) T7 RNA after the initiation complex formation. Symbols: ●, T7 RNA; ○, marker rRNA.*

TABLE 2. *Synthesis of active T7-specific RNA polymerase in an RNAse III-free, protein-synthesizing system in the presence of T7 RNA and T7 DNA*[a]

Nucleic acid added	Activity of T7-specific RNA polymerase synthesized (counts/min)
T7 RNA, uncleaved	3,817
T7 RNA, cleaved by RNase III	5,343
T7 DNA	4,355
T7 DNA + RNase III	3,542

[a] In vitro protein synthesis was carried out in an RNase III-free system (0.1 ml), as described in the legends of Fig. 4C and D, in the presence of 40 μg of T7 RNA (uncleaved or RNase III cleaved) synthesized in vitro and also in the presence of 4.6 μg of T7 DNA (7, 19). One of the T7 DNA-directed, protein-synthesizing systems received 1.8 μg of RNase III. After the reaction at 37 C for 45 min, 0.05-ml volumes were assayed for T7-specific RNA polymerase activity in the presence of rifampin as described previously (1, 20). The activity is expressed as amount of [^{3}H]UTP (2 μCi/200 μmol per 0.2-ml reaction mixture) incorporated into trichloroacetic acid-precipitable material after 20 min at 37 C.

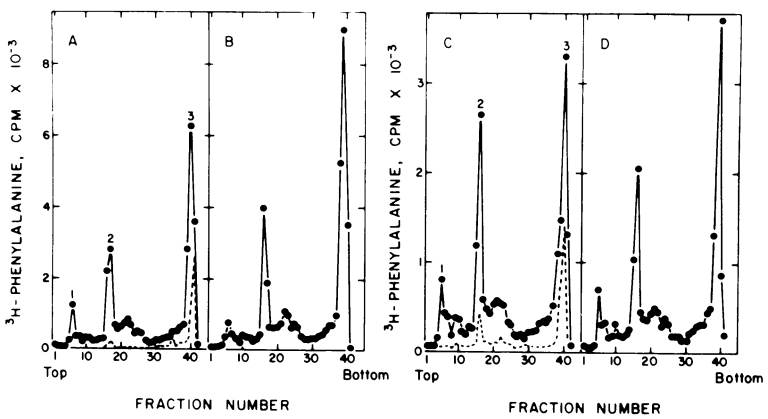

FIG. 4. *Acrylamide-SDS gel electrophoretic patterns of T7 proteins synthesized in vitro in the presence of uncleaved T7 RNA and RNase III-cleaved T7 RNA. (A and B) In vitro protein synthesis was carried out at 37 C for 45 min in a 0.05-ml reaction mixture containing the following: Tris-hydrochloride (pH 7.9), 54 mM; magnesium acetate, 10 mM; potassium acetate, 50 mM; NH_4Cl, 59 mM; dithiothreitol, 2.5 mM; ATP, 2 mM; CTP, GTP, and UTP, each at 0.5 mM; 20 amino acids, each at 0.1 mM; [^{3}H]phenylalanine (10.5 Ci/mmol), 5 μCi; stripped E. coli tRNA, 25 μg; phosphoenolpyruvate, 20 mM; spermidine, 2 mM; polyethyleneglycol, 2.5 mM; crude ribosomes, 250 μg; protein of S_{200} (RNase III free), 100 μg; and uncleaved T7 RNA synthesized in vitro (A) (15 μg) or RNase III-cleaved T7 RNA (B) (22.5 μg). (C and D) In vitro protein synthesis was carried out as for (A) and (B), except that crude ribosomes were replaced by RNase III-free, washed ribosomes (Table 1). The reaction mixture was diluted with 0.15 ml of buffer containing sodium dodecyl sulfate and boiled for 1 min, and then 0.08 ml of the solution was analyzed by 12.5% acrylamide-0.1% sodium dodecyl sulfate slab gel electrophresis as described previously (19). After staining and destaining, the gels were sliced and the radioactivity of gel slices was counted. Electrophoresis was from the left to right in the figure. The materials indicated as peaks 1, 2, and 3 were identified as T7-specific RNA polymerase, a mixture of T7 protein kinase and T7 ligase, and a mixture of gene 0.3 and gene 1.1 products. (A) In vitro system containing RNase III; uncleaved T7 RNA as messenger. The dotted line shows proteins synthesized without T7 RNA. (B) In vitro system containing RNase III; RNase III-cleaved T7 RNA as messenger. (C) In vitro system free of RNase III; uncleaved T7 RNA as messenger. (D) In vitro system free from RNase III; RNase III-cleaved T7 RNA as messenger.*

which had received the RNase III$^-$ character allowed completion of T7 phage development. Dunn and Studier further showed that the uncleaved T7 RNA is translatable both in vivo and in vitro yielding all T7 early proteins.

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