THE END GROUPS OF T7 mRNA

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Summary: Of the five T7 early mRNAs, only two (molecular weights, 1×10^6 and 0.2×10^6 daltons), were found to terminate in a guanosine triphosphate residue at the 5'-terminus. The amount of pppGp found indicates that several copies of the smaller RNA may be present. These two results suggest that the T7 early mRNAs cannot all be derived by cleavage from a single larger precursor. Uridine was found to be the predominant nucleoside at the 3'-terminus.

Bacterial mRNA is metabolically unstable, being degraded exponentially in the 5' to 3' direction with a half-life between 1 and 4 minutes (1-7). Recent studies have shown that decay rates of mRNAs from different segments of the try and lac operons differ (5,8,9). The mRNAs synthesized after infection of E. coli by T7 and T4 are unusually stable (10,11). The T7 mRNAs have half-lives of 15-20 minutes, but the decay rate of host mRNA is unaltered (12). With T4 infected cells, the phage-specific mRNAs are degraded at a rate 4-5 times slower than the host mRNA (11). The reasons for the difference in the decay rates between different mRNAs and the enhanced stability of the phage-specific mRNAs are unknown. It is possible that mRNA degradation is genetically determined and is governed by some structural or conformational feature in the mRNA, particularly at the 5'-terminus, since degradation commences at this terminus. Knowledge of the nucleotide sequence at the termini of these mRNAs may therefore contribute to an understanding of the mechanism of mRNA degradation. For characterization of the mRNAs, and as a preliminary to further studies, the end groups of T7 early mRNAs have been determined.

Methods: T7 and E. coli B/1 and BO11' were obtained from Dr. M. Chamberlin. T7 am 193, containing an amber mutation in gene 1, the phage RNA polymerase gene, was obtained from Dr. F. W. Studier. The phage and host strains were grown and maintained essentially as described by Studier (13).

^{32}P-labelled T7 mRNA: The host was grown in a low-phosphate, Tris-HCl buffered, pH 7.4, minimal salts medium containing 0.1% casamino acid. A culture of E. coli B/1 (10-20 ml) was aerated at 30° to a density of approximately 5 x 10^8 cells/ml before infection with either T7 or T7 am 193 at a multiplicity of 8 to 10. At 6 min after infection, carrier-free $^{32}PO_4^{\equiv}$ was added and the incubation was continued for an additional 6 min. The culture was poured onto ice water containing 0.005 M KCN and centrifuged for 5 min at 10,000 Xg; and the pellet was washed with 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl. After incubation of the pellet with lysozyme (80 μg/ml) at 0° for 5 min in 0.01 M Tris-HCl, pH 7.5, 0.45 M sucrose, 0.005 M EDTA, the cells were lysed by shaking with 1% sodium dodecyl sulfate for 5 min at room temperature. The lysate was extracted with 80% phenol, the combined aqueous layers extracted three times with chloroform-methanol (4:1), and the RNA precipitated with ethanol. The precipitated RNA was incubated with DNase (EC 3.1.-4.5, Worthington Biochemical Corp., grade DPFF) (20 μg/ml) in 0.1 M Tris-HCl, pH 7.5, at room temperature for 1 hr, and the RNA recovered by extraction with 80% phenol and precipitation with ethanol. Precipitation was carried out twice.

Gel Electrophoresis: The ^{32}P-labelled mRNAs were fractionated by electrophoresis according to the method of Loening (31) in 2.5% polyacrylamide gel cross-linked with ethylene diacrylate. After electrophoresis the gel was cut into slices of 1-mm width, and the ^{32}P-labelled RNA was recovered free of soluble gels by the method of Young and Young (14).

Results: The polyacrylamide gel electrophoresis patterns of the mRNAs synthesized after infection of E. coli B/1 with T7 and T7 am 193 are shown in Fig. 1. In the case of T7 (Fig. 1a) a minimum of nine discrete bands was obtained, a finding in agreement with the 11-13 bands observed by Summers, and Siegel and Summers (15,16). A much simpler RNA profile was obtained (Fig. 1b) when B/1, the non-permissive host, was infected with T7 am 193. Since the T7 RNA polymerase is absent, these are the T7 early mRNAs (16). The position of the

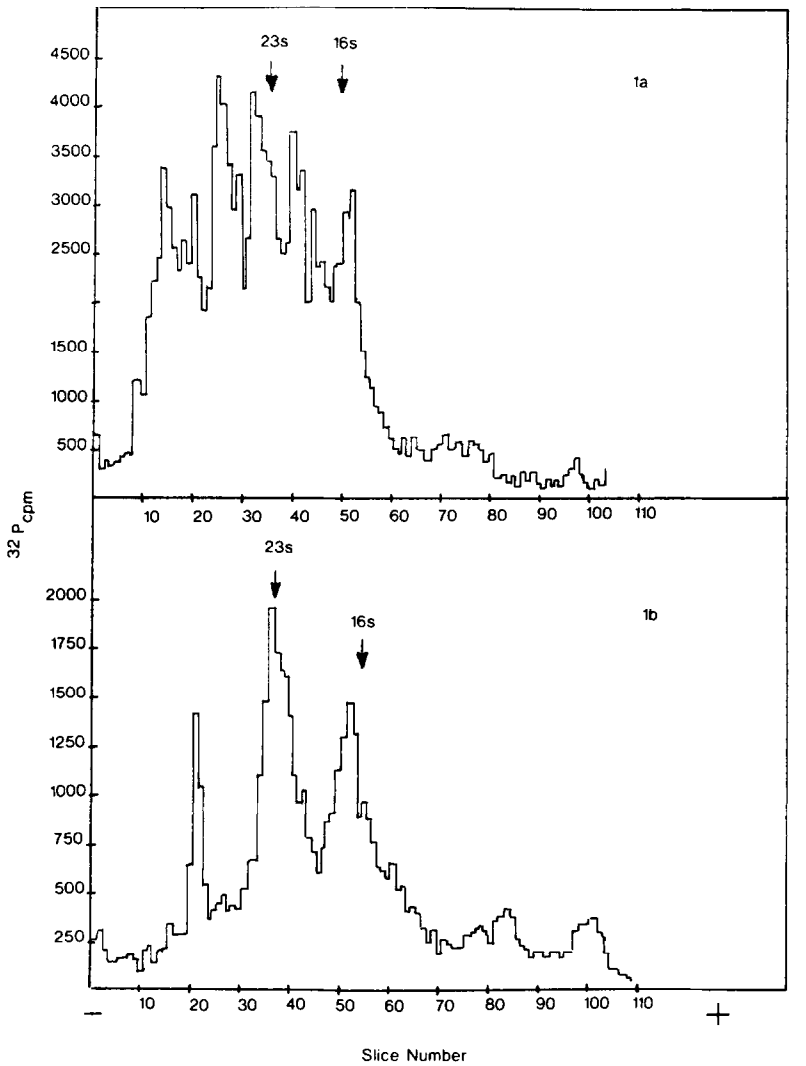

Figure 1: Polyacrylamide gel electrophoresis of T7 mRNA. E. coli B/1 was infected with T7 (1a) or T7 am 193 (1b) and six min after infection $^{32}PO_4^{\equiv}$ was added for six min. Electrophoresis of the isolated ^{32}P-labelled RNA was carried out in 2.5% polyacrylamide gel, 5 mA/tube as described by Loening (31); E. coli rRNA was added as marker prior to electrophoresis. After electrophoresis, the gel was stained with methylene blue, cut into 1 mm slices, dried, and radioactivity measured in a scintillation counter.

RNA peaks with respect to E. coli rRNA was highly reproducible. However, the size of the individual RNA peaks varied from experiment to experiment; the RNA band with the lowest mobility and the band with mobility slightly faster than E. coli. 16SRNA showed greater variation in size than the other RNA bands. Incubation of the mRNAs at room temperature for 1 hr with 90% dimethyl sulfoxide

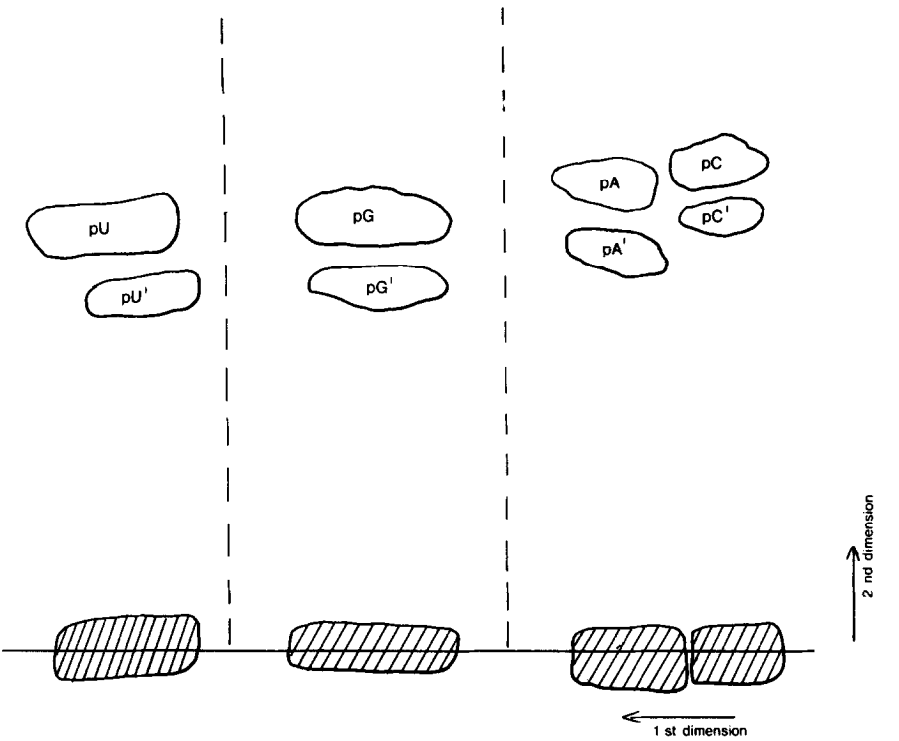

Figure 2: Diagram of a two dimensional electrophorogram of a snake venom digest of modified ^{32}P-labelled T7 am 193 mRNA. The ^{32}P-labelled RNA was modified and digested with snake venom phosphodiesterase as described (22). The digest and nucleoside trialcohol phosphate (pX') markers were spotted on a sheet of 3 MM paper and subjected to electrophoresis in pyridine acetate, pH 3.5 in the first dimension. After removal of the buffer, the electrophorogram was cut into sections (broken line), and each strip was subjected to electrophoresis in 0.05 M sodium borate, pH 8.5 in the second dimension. The shaded areas at the origin represent the positions of the spots after the first electrophoresis.

before gel electrophoresis did not eliminate or alter the relative sizes of any of the RNA bands. With E. coli rRNA and tobacco mosaic virus RNA as markers, the RNAs present can be assigned approximate molecular weights of 2.1×10^6, 1×10^6, 0.6×10^6, 0.46×10^6, 0.21×10^6 and 0.11×10^6.

The amount of contamination by ^{32}P-labelled host RNA should be low since low-phosphate medium was used, and $^{32}PO_4^{\equiv}$ was added 6 min after infection, when host RNA synthesis has essentially stopped (10,17,18). Hybridization to E. coli DNA was used to estimate the amount of ^{32}P-labelled host RNA present (19). Efficiency of hybridization was low because of the large amounts of unlabelled host RNA present. Only 0.16% and 0.23% of the input radioactivity

was retained when the ^{32}P-labelled RNA from T7 am 193-and T7-infected cells, respectively, were incubated with filters containing 40 ug E. coli DNA. When corrected for the binding to filters of ^{32}P-labelled RNA from control uninfected cells, the level of contamination by host RNA was estimated to be 5-10%.

The End Groups. The ^{32}P-labelled RNAs isolated from T7- and T7 am 193-infected E. coli, and from uninfected cells labelled with $^{32}PO_4^{\equiv}$ at the same cell density and for the same time interval, were hydrolyzed in 0.3 M KOH for 16 hr at 37°. Each of the alkaline hydrolysates was examined by electrophoresis in DEAE-paper in 7% formic acid 0.001 M EDTA. A triphosphorylated 5'-end group, if present, would have low mobility under these conditions and would be present at or near the origin, together with alkali-resistant oligonucleotides (32). A radioactive spot near the origin with a slower mobility than ppppG and pppA, added as markers, was present in the electrophorogram of each of the alkaline hydrolysates. Up to 45% of the radioactivity present in this spot was eluted by 2 M triethylammonium carbonate. The radioactive material eluted from the DEAE-paper was analyzed by electrophoresis on Whatman 3MM paper in 0.04 M sodium citrate, pH 3.3. A radioactive peak with the mobility expected for pppGp was present, and its identity was confirmed by enzymic degradation, as previously described (20,21). No material with the mobility of pppAp (20) was present in this electrophorogram. The amount of radioactivity present in the 5'-end group was 0.42%, 0.22%, and 0.13% respectively in the ^{32}P-labelled RNAs isolated from T7 am 193- and T7-infected cells and from control uninfected cells.

Each of the T7 am 193 mRNA species, separated by polyacrylamide gel electrophoresis, was hydrolyzed with alkali, and the hydrolysate examined by electrophoresis as described above. The pppGp end group was found only in the RNA bands of approximate size 1 and 0.2 x 10^6; the amount of radioactivity present was 0.08% and 0.4% respectively.

Table I

Percent of 3'-end groups in ^{32}P-Labelled RNA

	C	A	U	G
T7 mRNA	14	24	38	23
T7 am 193 mRNA	14	23	37	26
Uninfected E. coli Total RNA	20	24	20	35

The 3'-end group was identified by incubation of the ^{32}P-labelled RNA with $NaIO_4$ followed by reduction with $NaBH_4$; the 3'-terminus was released by snake-venom phosphodiesterase digestion of the modified ^{32}P-labelled RNA (22). Figure 2 shows that the internal nucleotides (pX) were clearly separated by two-dimensional electrophoresis from the 3'-end groups which was present in the snake-venom digest as a ^{32}P-labelled nucleoside trialcohol monophosphate (pX'). The 3'-end groups found in the T7 mRNAs are listed in Table 1; uridine was the predominant 3'-terminal nucleoside.

Discussion: The five T7 early mRNAs, listed in genetic order from the left end have molecular weights of 0.24, 0.70, 1.15, 0.24 and 0.47 x 10^6 daltons, when measured by migration rates in polyacrylamide gels; lower values have been obtained by heteroduplex mapping (18,23,24). RNA species of these sizes, together with one of 2 x 10^6 daltons, have been found in the present work. The origin and function of this large RNA is not known, although other workers have also observed a large RNA (18,25). The radioactivity present in the pppGp ends of the ^{32}P-labelled RNAs of size 1 x 10^6 and 0.2 x 10^6 should amount to 0.13% and 0.66%, respectively, of the total radioactivity. An equimolar mixture of the five ^{32}P-labelled T7 early mRNAs in which only two species, 1 x 10^6 and 0.2 x 10^6, contained a 5'-triphosphate end would be expected to have $<$ 0.1% of the

total radioactivity associated with the 5'-end group. The finding that 0.42% of the radioactivity of ^{32}P-labelled RNAs from T7 am 193-infected cells is present in the 5'-end group suggests that several copies of the smaller RNA species are present, even though the T7 am 193 RNAs are probably not uniformally labelled. The presence of several copies of the small RNA has also been reported by Summers et al. (18).

Although five RNA species corresponding to the five T7 early genes are obtained *in vivo*, transcription *in vitro* of the T7 early genes by *E. coli* RNA polymerases produces only a single transcript of 2.2×10^6 daltons (26-29). The RNAs found *in vivo* are thought to be released during transcription by rho factor, or by a post-transcriptional modification of the large RNA (27,28). Evidence for a post-transcriptional event has been provided by Dunn and Studier (29) who found a sizing factor which specifically cleaved the large RNA transcript into five RNA species of the same size as the T7 early mRNAs and a fragment from the initiator region. The T7 early mRNAs therefore should not contain a 5'-triphosphate, but the fragment from the initiator region might. RNAs from the early region arising by rho-mediated terminations should have a 5'-triphosphate end group.

Alternatively, the T7 early mRNAs may be produced by initiation and termination of transcription at each early gene (30). Recently, transcription and termination sites have been detected at the ends of the early genes, 0.7 and 1 (30). Their respective RNA transcripts of size 0.7 and 1.15×10^6 daltons, as well as RNA products of the other three early genes, should, unless modified, contain a 5'-triphosphate end group. The present finding of a 5'-triphosphate group in the RNAs of approximately 1 and 0.2×10^6 daltons, and the apparent large amounts of the latter species tend to support this but not the post-transcriptional model (29). However, some or all of the small RNAs terminating in a 5'-triphosphate group may be untranslated initiator fragments (29), products of weak or minor initiation events (29), random cleavage products of larger RNAs, or small RNAs present in early T7 mRNAs of undefined function

(18,23,24,29), and are not the mRNA products of the early genes, 0.3 and 1.1. In this event, the T7 early mRNAs may be present in equimolar amounts, as required by the post-transcriptional model; the absence of a 5'-triphosphate end group in some of the mRNAs is also compatible with this model. The presence of a 5'-triphosphate group in the RNA of 1×10^6 daltons means that not all T7 early mRNAs can be produced by post-transcriptional modification. Both mechanisms may operate in vivo for the generation of the five early T7 mRNAs

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