# Characterization of Ribonucleic Acid Transcribed *in Vitro* on Phage $\phi$80 Deoxyribonucleic Acid*

Toshio Okamoto, Masahiro Sugiura, and Mituru Takanami

ABSTRACT: The characterization of the RNA species transcribed *in vitro* on phage $\phi$80 DNA by *Escherichia coli* RNA polymerase was based on the finding that the RNA chains were initiated predominantly with purine ribonucleoside triphosphates.

RNA initiated with pppA-- was homogeneous with regard to the size and starting nucleotide sequence: the size was about 10 S and the sequence was pppApUpNpG--(N = C, U, or A). With enzyme lacking the $\sigma$ factor, the starting sequence became heterogeneous. The addition of a termination factor $\rho$ to the RNA-synthesizing mixture did not affect the size. RNA initiated with pppG-- was classified into two major classes starting with sequences of pppGpC-- and pppGpApU--. On band centrifugation, these RNA components were separated into four peaks of about 4, 13, 26, and 37 S. When RNA had been synthesized in the presence of the $\rho$ factor, the 26S and 37S peaks disappeared and instead, two major peaks were observed at the 6S and 13S regions. The 4S RNA species was present at the front shoulder of the 6S peak. The starting sequences of the 4S and 6S components were pppGpC--- and that of the 13S component was pppGpApU---.

It has been shown that the RNA molecule synthesized by RNA polymerase contains a nucleoside triphosphate at the 5′ terminus and grows by the sequential addition of ribonucleotides to the 3′-hydroxyl group of the 3′ end (Maitra and Hurwitz, 1965; Bremer *et al.*, 1965). The finding has made it possible to label the 5′ termini of RNA with radioactive phosphate. Applying this terminal labeling method, we have attempted to determine the starting nucleotide sequences and size of RNA transcribed on various DNA templates. Since translation of the genetic message also proceeds from the 5′ to 3′ end of mRNA (Salas *et al.*, 1965; Thach *et al.*, 1965), there is a possibility that the 5′-terminal region of mRNA contains a specific sequence responsible for the initiation of protein synthesis. As the other possibility, Imamoto (1968) has presented evidence suggesting that transcription is initiated at a site before the operator region of Trp-operon. If so, the starting sequence of RNA may reflect such the sequence of DNA responsible for the regulation of RNA transcription. As reported previously (Sugiura *et al.*, 1969; Okamoto *et al.*, 1969), we first characterized the RNA species transcribed on the doubly closed replicative form DNA (RF-I DNA) of phage fd by *E. coli* RNA polymerase, and found that three different sizes of RNA starting with the sequences of pppApUpG--, pppGpUpA--, and pppGpUpU-- were synthesized on the template. Two of these sequences are proposed initiation codons for protein synthesis. In this paper, we report the starting sequences and size of RNA transcribed on phage $\phi$80 DNA by *E. coli* RNA polymerase.

## Materials and Methods

*Phage and Phage DNA.* Phage $\phi$80 was prepared by mitomycin induction (1.5 $\mu$g/ml) of lysogenic bacterium: W3350-($\phi$80). The phage was purified from the lysate by the method of Kaiser and Hogness (1960). DNA was extracted from the purified phage by the phenol method as described by Richardson *et al.* (1964). After the phenol treatment, the DNA solution was dialyzed extensively against 0.2 mM EDTA–0.5 mM phosphate buffer (pH 7.5) to remove contaminated phenol. DNA thus prepared was stored at 0°.

*RNA Polymerase.* This was prepared from *E. coli* A19 by the method of Chamberlin and Berg (1962) and purified through band centrifugation on sucrose density gradients (Sugiura *et al.*, 1969). The activity peak region with about 22 S was collected. The specific activity was about 2500 units/mg. The enzyme contained no detectable endonuclease activity. The plaque-forming abilities of fd DNA and f2 RNA on lysozyme spheroplasts were not affected by incubation for 1 hr at 37° under the conditions used for RNA synthesis.

*RNA Polymerase Core and $\sigma$ Factor.* RNA polymerase prepared as above was fractionated into the core enzyme and $\sigma$ factor by phosphocellulose column chromatography, according to the method of Burgess *et al.* (1969).

*$\rho$ Factor.* The $\rho$ factor was prepared from *E. coli* A19 cells as described by Roberts (1969). Frozen cells were ground with quartz sand and extracted with Roberts' buffer. The extract was treated with pancreatic DNase and then centrifuged for 2 hr at 100,000*g*. Following the ammonium sulfate fractionation, the fraction containing $\rho$ was chromatographed on phosphocellulose. Fractions around the peak of depression were collected, and chromatographed on DEAE-cellulose. The peak of depression was collected, rechromatographed on DEAE-cellulose and used for experiments. The $\rho$ fraction contained no detectable endonuclease activity, when it was assayed by the infectivities of fd DNA and f2 RNA on lysozyme spheroplasts. The effect of the $\rho$ fraction on $\phi$80 DNA primed RNA synthesis is illustrated in Figure 1. In accordance with the observation by Roberts (1969), total RNA synthesis was depressed by $\rho$ to a plateau value. In experiments where the effect of $\rho$ on size of RNA

* From the Institute for Chemical Research, Kyoto University, Uji, Kyoto-fu, Japan. *Received January 12, 1970.*

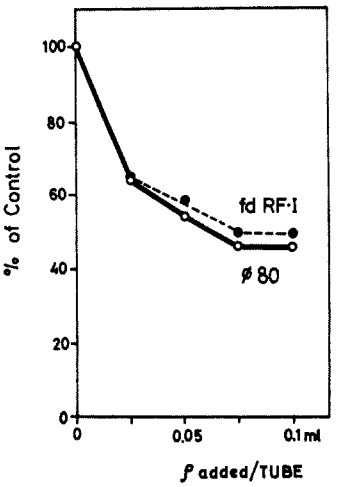


FIGURE 1: Effect of the $\rho$ factor on $\phi$80 DNA-primed RNA synthesis. Each reaction mixture (0.5 ml) contained 8 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 0.4 mM each of CTP, UTP, GTP, and [$^3$H]ATP (1000 cpm/m$\mu$mole), 16 $\mu$g of DNA, 8 $\mu$g of enzyme, and indicated amounts of the $\rho$ fraction. Following incubation for 20 min at 37°, each reaction was terminated by cold 10% trichloroacetic acid–20 mM pyrophosphate. The precipitates were collected on Millipore filters, washed with cold trichloroacetic acid, and the radioactivity was assayed.

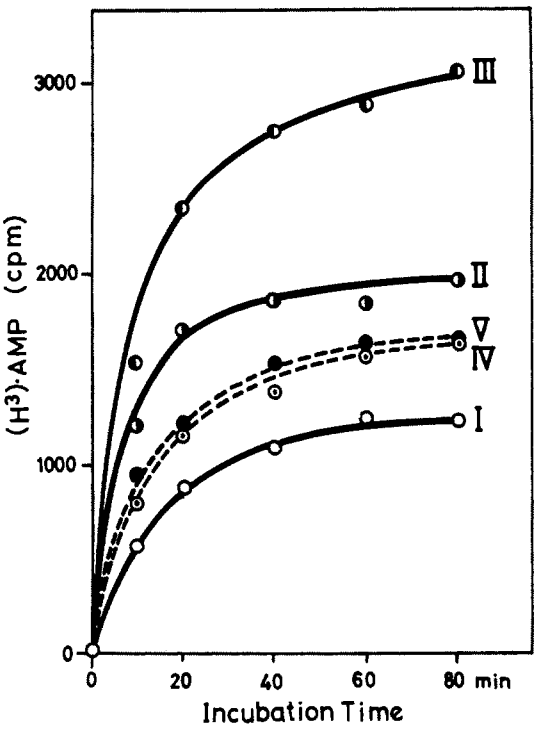


FIGURE 2: Time course of [$^3$H]AMP incorporation into RNA. The reaction mixture (0.5 ml) contained 8 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 0.4 mM each of CTP, UTP, GTP, and [$^3$H]ATP (1000 cpm/m$\mu$mole), and the following amounts of DNA and enzyme. Series I, II, and III contained 8 $\mu$g each of DNA and 8, 16, and 24 $\mu$g of enzyme, respectively. Series IV and V contained 8 $\mu$g each of enzyme and 16 and 24 $\mu$g of DNA, respectively. Following incubation at 37°, each reaction was terminated, and [$^3$H]AMP incorporated was assayed as in the legend to Figure 1.

was examined, 0.2 ml of the $\rho$ fraction was added to a 1-ml RNA-synthesizing mixture.

*Conditions for RNA Synthesis.* The rate of RNA synthesis was determined at different ratios of template to RNA polymerase in reaction mixtures containing 8 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, and 0.4 mM each of the four ribonucleoside triphosphates. As shown in Figure 2, the rate and extent of RNA synthesis was markedly increased with the enzyme concentration. Upon increasing the amount of template, however, the rate became nearly constant. Unless otherwise noted, RNA synthesis was carried out under two extreme conditions: one was with excess template (curve IV in Figure 2) and the other with excess enzyme (curve III in Figure 2).

Figure 3 shows the effect of KCl concentration on RNA synthesis. Maximum RNA synthesis with $\phi$80 DNA was observed at around 0.05 M KCl, whereas RNA synthesis primed by T4 DNA and fd RF-I DNA was rather stimulated at relatively high salt concentration. Unless otherwise noted, RNA synthesis in the following experiments was performed in the presence of 0.04 M KCl.

*Synthesis of RNA Starting with [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP.* As described previously (Sugiura *et al.*, 1969), the rate of RNA synthesis was very low at low substrate concentration, but the number of initiation did not vary very much with the substrate concentration. To label the 5′ termini of RNA with $^{32}$P of high specific activity and chase the labeled RNA chains, therefore, RNA chains were first initiated with nucleoside [$\gamma$-$^{32}$P]triphosphates at a low substrate concentration, and then elongated by adding excess amounts of nonlabeled nucleoside triphosphates. The detailed conditions for synthesis were as follows: 4 $\mu$M of either [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP and 40 $\mu$M each of three other nucleoside triphosphates were added to reaction mixtures containing 8 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, and indicated amounts of DNA and enzyme. Following incubation for 5 min at 37°, CTP, UTP, GTP, and [$^3$H]ATP were added to 0.4 mM, and incubation was continued. At the end of incubation, the reaction mixture was transferred to an ice bath, and the RNA fraction was extracted by treatment with 80% phenol. *E. coli* rRNA was added as carrier and the mixture was passed through a Sephadex G-100 column (1 × 20 cm for a 1-ml solution), equilibrated with 80% formamide–0.05 M Tris-HCl (pH 7.6). The labeled RNA fraction eluted was collected, dialyzed against 10% formamide–0.14 M NaCl–0.02 M Tris-HCl (pH 7.6), and submitted to the size determination and sequence analysis.

*Analysis of the Starting Nucleotide Sequences.* RNA labeled with either [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP was synthesized as above. Incubation after adding excess substrates was terminated by 10 min. The synthesized RNA was extracted, isolated using a Sephadex G-100 column, and then precipitated with carrier rRNA by adding cold ethanol. The precipitate was dissolved in a small volume of 0.1 M Tris-HCl (pH 7.6) and hydrolyzed with either T1 RNase or pancreatic RNase. The hydrolysate was charged on a DEAE-Sephadex column (A25, 0.6 × 20 cm) and eluted with a NaCl gradient formed in 7 M urea (pH 7.8). The detailed conditions for hydrolysis and chromatography were described elsewhere (Takanami, 1967). The separation of di- and trinucleotides with different sequences was performed by chromatography on Dowex 1-X2 columns (Sugiura *et al.*, 1969).

TABLE I: Incorporation of Nucleoside [$\gamma$-$^{32}$P]Triphosphates into RNA Synthesized on $\phi$80 DNA.[a]

| Template ($\mu$g/tube) | Enzyme ($\mu$g/tube) | Nucleoside [$\gamma$-$^{32}$P]Triphosphates Incorporated into RNA ($\mu\mu$moles) | | | |
|---|---|---|---|---|---|
| | | ATP | GTP | CTP | UTP |
| 24 | 8 | 0.98 | 1.08 | 0.08 | 0.07 |
| 16 | 8 | 0.72 | 1.15 | 0.11 | 0.05 |
| 8 | 8 | 0.52 | 1.25 | 0.11 | 0.08 |
| 8 | 16 | 0.58 | 1.83 | 0.15 | 0.09 |
| 8 | 24 | 0.75 | 2.16 | 0.16 | 0.08 |

[a] The reaction mixture (0.5 ml) contained 8 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 4 $\mu$M of a nucleoside [$\gamma$-$^{32}$P]triphosphate, 40 $\mu$M each of three other nucleoside triphosphates, and indicated amounts of template and enzyme. After incubation for 10 min at 37°, $^{32}$P incorporated into RNA was assayed as in the legend to Figure 1.

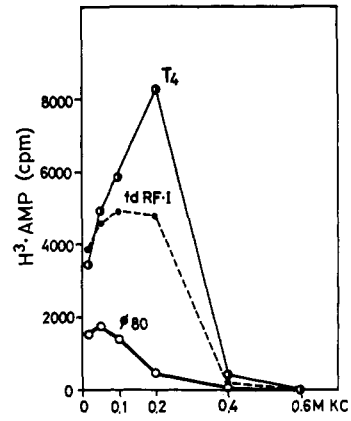


FIGURE 3: Effect of KCl concentration on RNA synthesis. The composition and volume of the reaction mixture were similar to those in series IV of Figure 2, except for the concentration of KCl. Following incubation for 20 min at 37°, [$^3$H]AMP incorporated was assayed as in the legend to Figure 1.

*Band Centrifugation.* This was performed in the presence of 10% formamide to prevent aggregation. Sucrose density gradients, 5–20% in 10% formamide–0.14 M NaCl–0.02 M Tris-HCl (pH 7.6), were formed in Spinco SW25 tubes. About 1 ml of the labeled RNA was layered on the gradient and centrifuged for 16 hr at 25,000 rpm and 5°. Drops were collected from the tube bottom. After fractionation, the tube bottom was washed with a small volume of water, and the washings were combined with the first fraction (tube 1 in figures). Carrier albumin (0.1 mg), followed by two volumes of cold 10% trichloroacetic acid, were added to each tube. Precipitates formed were collected on Millipore filters, and the radioactivity was assayed. In some experiments, 0.5 ml each of fractions was transferred to a vial, containing 6 ml of ethylene glycol monomethyl ether and 10 ml of 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene, and the radioactivity was assayed.

*Other Materials.* The methods of preparing T4 DNA and fd RF-I DNA were those of Richardson *et al.* (1964) and Sugiura *et al.* (1969). Ribonucleoside [$\gamma$-$^{32}$P]triphosphates with a specific activity of about $10^{10}$ cpm/$\mu$mole were synthesized according to Glynn and Chappell (1964). Alkaline phosphatase was purified from *E. coli* A19 cells as previously described (Takanami, 1967). Generally labeled [$^3$H]ATP and [$^3$H]GTP were purchased from Schwarz BioResearch, Inc.

## Results

*Incorporation of Nucleoside [$\gamma$-$^{32}$P]Triphosphates into RNA.* Incorporation of $^{32}$P from each of the four nucleoside [$\gamma$-$^{32}$P]-triphosphates into RNA was measured at different ratios of template to enzyme. As has been observed with a variety of DNA templates (Maitra and Hurwitz, 1965; Krakow and Horsley, 1967; Sentenac *et al.*, 1968), RNA chains formed on $\phi$80 DNA were also initiated predominantly with purine nucleoside triphosphates( Table I). Little incorporation of [$\gamma$-$^{32}$P]-CTP and [$\gamma$-$^{32}$P]UTP was observed under the conditions used. It was noted, however, that with excess template the number of the ATP termini formed became roughly equal to that of the GTP termini, whereas the ratio of ATP to GTP incorporated into the 5′ end of RNA was about 1:3 at the excess enzyme condition.

*Band Centrifugation Analysis of Synthesized RNA.* RNA STARTING WITH pppG--. RNA synthesis was initiated with [$\gamma$-$^{32}$P]GTP by a short incubation in the reaction mixture containing low substrate concentration and excess template. The concentration of the four nucleoside triphosphates including [$^3$H]ATP was then increased to promote RNA chain elongation. At 5, 10, and 20 min after the addition of excess substrates, each reaction was terminated. The synthesized products were extracted, and analyzed by band centrifugation in the presence of formamide. The sedimentation profiles are shown in Figure 4, in which $^{32}$P corresponds to the initiating nucleotide and $^3$H to the chain nucleotides, respectively. At 5 min, a few $^{32}$P peaks appeared at relatively lighter regions. After further incubation, $^{32}$P was found

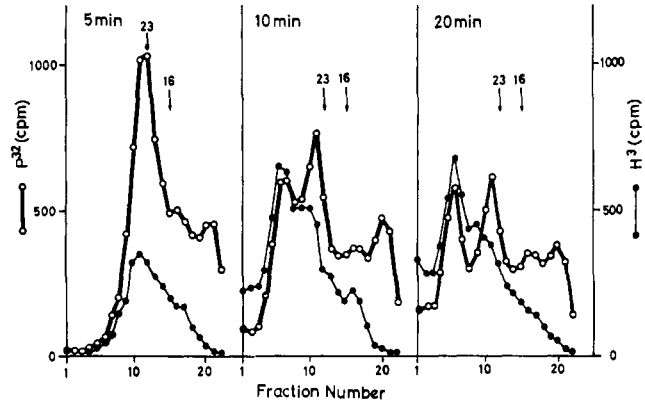


FIGURE 4: Sedimentation analysis of RNA starting with pppG- -. RNA-synthesizing mixtures (1 ml each) containing [$\gamma$-$^{32}$P]GTP and excess template were prepared as in the text. Following addition of excess substrates including [$^3$H]ATP (2000 cpm/m$\mu$mole), each reaction was terminated at 5, 10, and 20 min. The RNA fraction was extracted and analyzed by band centrifugation, as in the text. The arrows indicate the positions of *E. coli* rRNA as markers.

TABLE II: Recovery of $^{32}P$ in the Fragments Produced from T1 RNase and Pancreatic RNase Hydrolysates of RNA Labeled with [$\gamma$-$^{32}P$]GTP and [$\gamma$-$^{32}P$]ATP.[a]

| [$\gamma$-$^{32}P$]GTP-RNA | | [$\gamma$-$^{32}P$]ATP-RNA | | | |
|---|---|---|---|---|---|
| Pancreatic RNase | | T1 RNase | | Pancreatic RNase | |
| Fragments | % of Total | Fragments[b] | % of Total | Fragments | % of Total |
| pppGpPyp | 41 | pppApGp | 6 | pppApPyp | 93 |
| pppGpPupPyp | 56 | pppApNpGp | 6 | pppApPupPyp | 6 |
| pppGp(Pup)$_2$Pyp | 3 | pppAp(Np)$_2$Gp | 73 | pppAp(Pup)$_2$Pyp | 1 |
| $\geqq$pppGp(Pup)$_3$Pyp | 1 | $\geqq$pppAp(Np)$_3$Gp | 10 | $\geqq$pppAp(Pup)$_3$Pyp | 0 |

[a] RNA labeled with [$\gamma$-$^{32}P$]GTP was hydrolyzed with pancreatic RNase and RNA labeled with [$\gamma$-$^{32}P$]ATP with either T1 RNase or pancreatic RNase, and resulting $^{32}P$ fragments were separated by DEAE-Sephadex column chromatography, as in the legend to Figure 6. Distribution of $^{32}P$ in fractions was determined. [b] N = C, U, or A.

distributed at four principal regions. When the approximate S values were estimated from the relative positions of rRNA as marker, the values at the peak positions were roughly 4, 13, 26, and 37 S, respectively. The $^{3}H$ peaks were also coincident with the $^{32}P$ peaks, although the ratio of $^{3}H$ to $^{32}P$ increased with size of RNA. Incubation was carried out for an additional 20 min. However, there was no significant change in the sedimentation profile.

RNA was synthesized with excess enzyme, and the sedimentation profile of the synthesized product was compared with those of Figure 4, in which RNA synthesis was carried out with excess template. The patterns obtained were essentially identical with those of Figure 4. It was noted, however, that the relative height of the 4S and 26S peaks increased considerably.

RNA STARTING WITH pppA--. [$\gamma$-$^{32}P$]ATP was added to the reaction mixtures in place of [$\gamma$-$^{32}P$]GTP in the above experiments, and similar experiments were carried out. As shown in Figure 5, RNA starting with pppA-- gave a single peak of about 10 S, in sharp contrast to RNA starting with pppG--. The sedimentation profile of RNA synthesized with excess enzyme was also identical with those of Figure 5, in which RNA was synthesized with excess template.

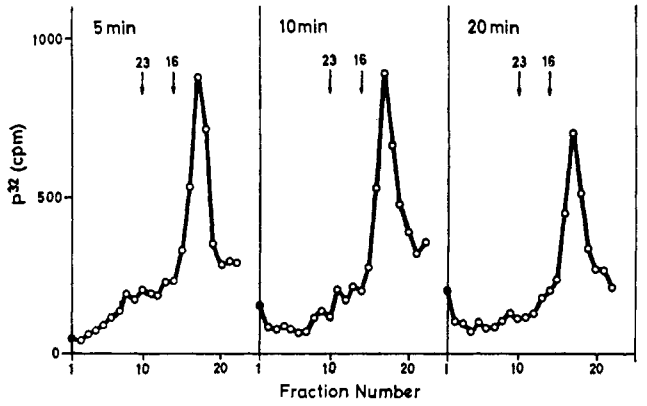


FIGURE 5: Sedimentation analysis of RNA starting with pppA- - -. In place of [$\gamma^{32}P$]GTP in the experiment of Figure 4, [$\gamma^{32}P$]ATP was added to the reaction mixtures. At 5, 10, and 20 min following addition of excess substrates, each reaction was terminated, and the synthesized products were analyzed by band centrifugation. The arrows indicate the positions of rRNA as markers.

*Analysis of the Starting Nucleotide Sequences.* RNA STARTING WITH pppG--. RNA labeled with [$\gamma$-$^{32}P$]GTP was synthesized under excess template conditions and hydrolyzed with pancreatic RNase. The resulting $^{32}P$ fragments were separated by DEAE-Sephadex column chromatography using the conditions appropriate for oligonucleotide separation. The hydrolysate produced major $^{32}P$ peaks at the pppGpPyp and pppGpPupPyp regions (Figure 6A). Recovery of $^{32}P$ in these two fragments was about 97% of total $^{32}P$ applied (Table II).

To identify the species of Py in pppGpPyp and Pu and Py in pppGpPupPyp, RNA was synthesized in the presence of [$\gamma$-$^{32}P$]GTP and [$^{3}H$]GTP. The conditions used for synthesis were similar to those for labeling the 5′ termini, but [$^{3}H$]GTP of high specific activity was added together with [$\gamma$-$^{32}P$]GTP. The synthesized RNA was extracted and hydrolyzed with pancreatic RNase, and the resulting pppGpPyp and pppGpPupPyp fractions were isolated by DEAE-Sephadex column chromatography. Since these fractions also contained fragments produced from the internal region of RNA, the respective fractions were treated with alkaline phosphatase and rechromatographed on DEAE-Sephadex columns. By this treatment, the terminal fragments released four orthophosphates (pppGpPyp → GpPy and pppGpPupPyp → GpPupPy) and separated from the fragments from the internal region of RNA which lost only one orthophosphate ((Pup)$_n$Pyp → (Pup)$_n$Py). The detailed conditions for alkaline phosphatase treatment and chromatography were described previously (Sugiura *et al.*, 1969). To the [$^{3}H$]GpPy fraction thus obtained, GpC and GpU were added as markers, and the mixture was chromatographed on a Dowex 1 column. About 80% of $^{3}H$ applied was found at the GpC peak. To the [$^{3}H$]GpPupPy fraction, GpApC, GpApU, GpGpC, and GpGpU were added as markers, and the mixture was chromatographically resolved. About 95% of $^{3}H$ applied was found at the GpApU region.

It was concluded that the major components starting with pppG-- had the sequences of pppGpC-- and pppGpApU--.

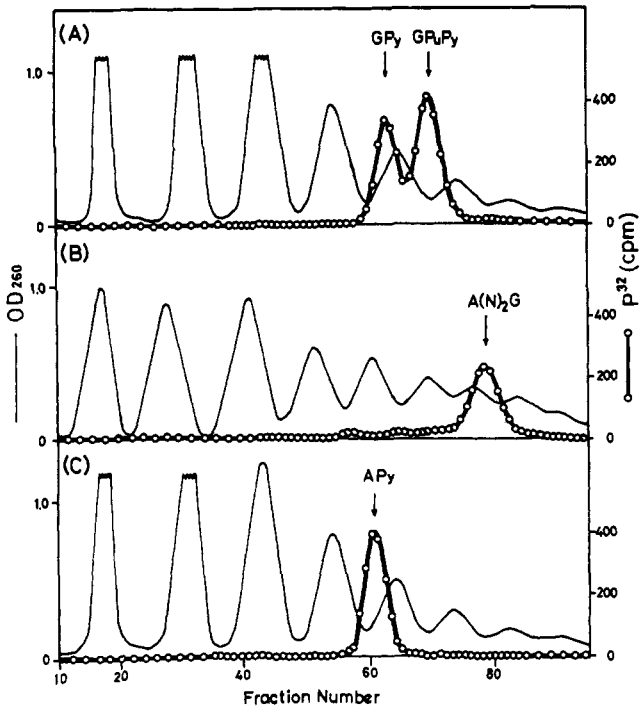


FIGURE 6: DEAE-Sephadex column chromatography of T1 RNase and pancreatic RNase hydrolysates of RNA labeled with [$\gamma$-$^{32}$P]-GTP and [$\gamma$-$^{32}$P]ATP. RNA labeled with [$\gamma$-$^{32}$P]GTP and [$\gamma$-$^{32}$P]-ATP were synthesized with excess template as in the text. Incubation after adding excess substrates was for 10 min at 37°. Non-radioactive RNA was added to provide markers, and the mixtures were hydrolyzed with either T1 RNase or pancreatic RNase. The hydrolysates were charged onto DEAE-Sephadex columns (0.6 × 20 cm) and chromatographed with a linear gradient from 0.08 M NaCl–7 M urea to 0.4 M NaCl–7 M urea (pH 7.8). (A) Pancreatic RNase hydrolysate of [$\gamma$-$^{32}$P]GTP-RNA, (B) T1 RNase hydrolysate of [$\gamma$-$^{32}$P]ATP-RNA, and (C) pancreatic RNase hydrolysate of [$\gamma$-$^{32}$P]ATP-RNA.

RNA labeled with [$\gamma$-$^{32}$P]GTP was synthesized with excess enzyme, hydrolyzed with pancreatic RNase, and $^{32}$P fragments produced were analyzed as above. The major terminal fragments identified were identical with those produced from RNA made with excess template. It was noted, however, that the ratio of pppGpC-- to pppGpApU-- was increased when RNA was synthesized with excess enzyme. Consistent with this was the observation that the relative amount of the 4S and 26S components increased considerably by synthesizing RNA with excess enzyme and that, as described below, RNA starting with pppGpC-- was found in these sized components.

As indicated in Figure 4, RNA starting with pppG-- was separated into four peaks by band centrifugation. Accordingly, RNA labeled with [$\gamma$-$^{32}$P]GTP was synthesized and fractionated by band centrifugation. The $^{32}$P peak regions were collected as indicated in Figure 7. Following hydrolysis with pancreatic RNase, $^{32}$P fragments produced from each peak were analyzed. The results of analyses are shown in Table III, where distribution of the indicated terminal fragments was estimated from the proportion of the four peaks and the ratios of the terminal fragments in each peak. The component at the 4S peak was initiated predominantly with pppGpC-- and those

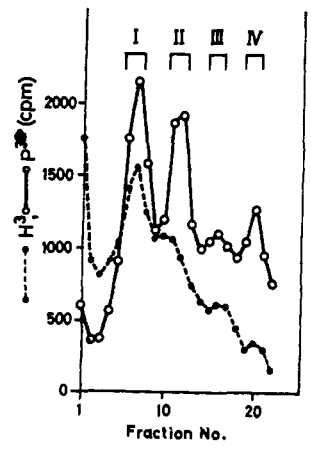


FIGURE 7: Fractionation of RNA starting with pppG-- by band centrifugation. RNA labeled with [$\gamma$-$^{32}$P]GTP was prepared with excess template, as in the text. Incubation after adding excess substrates was for 20 min. The labeled RNA was centrifuged on a sucrose density gradient, and the $^{32}$P peak regions, indicated in the figure by arrows, were collected, and used for experiments.

at the 13S and 37S peaks with pppGpApU--. The 26S peak appeared to contain two components starting with pppGpC-- and pppGpApU--. As described in the following section, however, the distribution of the terminal label was markedly affected by the addition of the $\rho$ factor to the RNA-synthesizing mixture.

RNA STARTING WITH pppA--. RNA labeled with [$\gamma$-$^{32}$P]ATP was synthesized using excess template conditions, and their starting sequences were analyzed as in the above experiments. The T1 RNase hydrolysate produced the major $^{32}$P peak at the pppApNpNpGp region (N = C, U, or A), and the pancreatic RNase hydrolysate at the pppApPyp region (Figure 6B,C). Recoveries of $^{32}$P in these major fragments are shown in Table II.

To identify the species of Py at the second position, RNA

TABLE III: Distribution of the pppGpCp and pppGpApUp Fragments.[a]

| | % of G-Terminal Label in Fractions | | | |
|---|---|---|---|---|
| | I (37 S) | II (26 S) | III (13 S) | IV (4 S) |
| Total G termini | 34 | 31 | 18 | 17 |
| pppGpCp | 2.5 | 11.6 | 3.7 | 13.4 |
| pppGpApUp | 30.9 | 17.6 | 12.9 | 2.6 |

[a] RNA labeled with [$\gamma$-$^{32}$P]GTP was centrifuged on a sucrose density gradient and the $^{32}$P peak regions were collected as indicated in Figure 7. Each fraction was hydrolyzed with pancreatic RNase, and $^{32}$P fragments produced were chromatographically resolved. Distribution (per cent) of the indicated terminal fragments in fractions was estimated from the proportion of the four peaks and the ratios of the fragments in each peak.

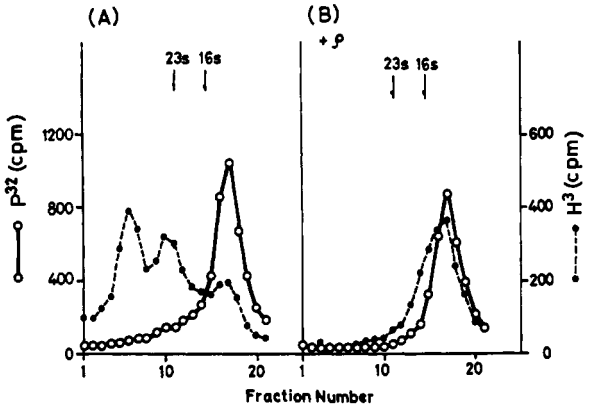


FIGURE 8: Sedimentation profiles of RNA starting with pppA--, synthesized with and without $\rho$. Two RNA-synthesizing mixtures (1 ml each) containing [$\gamma$-$^{32}$P]ATP and excess template were prepared as in the text. To one tube was added 0.2 ml of the $\rho$ fraction. At 20 min after adding excess substrates including [$^{3}$H]ATP (2000 cpm/m$\mu$mole), the reaction was terminated. The labeled RNA was analyzed by band centrifugation. (A) RNA synthesized in the absence of $\rho$ and (B) RNA synthesized in the presence of $\rho$.

TABLE IV: The Starting Sequences of [$\gamma$-$^{32}$P]ATP-Labeled RNA Synthesized by RNA Polymerase with and without $\sigma$ Factor.[a]

| Fragments | % of Total: Core Enzyme | % of Total: Core Enzyme + $\sigma$ |
|---|---|---|
| pppApPyp | 43.5 | 86.9 |
| pppApPupPyp | 10.3 | 2.6 |
| pppAp(Pup)$_2$Pyp | 7.5 | 0.9 |
| $\geqq$pppAp(Pup)$_3$Pyp | 38.7 | 9.6 |

[a] RNA labeled with [$\gamma$-$^{32}$P]ATP was synthesized as in the text, except that fractionated components of RNA polymerase were used. The amounts of core enzyme and $\sigma$ factor added per 1-ml reaction mixture were 10 and 5 $\mu$g, respectively. Incubation after adding excess substrates was for 10 min. The synthesized products were hydrolysed with pancreatic RNase, and $^{32}$P fragments produced were separated by DEAE-Sephadex column chromatography.

was synthesized in the presence of [$\gamma$-$^{32}$P]ATP and [$^{3}$H]ATP The labeled RNA was hydrolyzed with pancreatic RNase, and the pppApPyp fraction was separated. Following treatment with alkaline phosphatase, the [$^{3}$H]ApPy fraction was isolated. ApC and ApU were added as markers, and the mixture was chromatographed on a Dowex 1 column. About 90% of $^{3}$H applied was recovered at the ApU region.

It was concluded that the major component of RNA starting with pppA-- had the sequence of pppApUpNpG--.

*The Starting Sequences of RNA Synthesized with Enzyme Lacking $\sigma$.* It has been shown that RNA polymerase $\sigma$ factor is required for the initiation of RNA synthesis (Burgess *et al.*, 1969; Travers, 1969; Travers and Burgess, 1969; Summers and Siegel, 1969). Without $\sigma$ factor, RNA polymerase results in random initiation along the template (Bautz *et al.*, 1969). Although the enzyme lacking $\sigma$ could initiate RNA synthesis with purine ribonucleoside triphosphates, the synthesized products differed in their subsequent nucleotide sequences from those formed by the complete enzyme (Sugiura *et al.*, 1970).

To examine the effect of the $\sigma$ factor on the starting sequences of RNA transcribed on $\phi$80 DNA, RNA was synthesized by RNA polymerase with and without $\sigma$ factor, and their starting sequences were compared. As has been observed with T4 DNA (Burgess *et al.*, 1969), $\phi$80 DNA was also poor template for the enzyme lacking $\sigma$. Under the conditions used for the terminal labeling, the termini formed with the core enzyme were less than 10% of those formed with the complete enzyme. Addition of $\sigma$ to the core enzyme restored the RNA-synthesizing ability. RNA labeled with [$\gamma$-$^{32}$P]ATP was synthesized with the core enzyme and hydrolyzed with pancreatic RNase. The resulting $^{32}$P fragments were chromatographically resolved. As shown in Table IV, less than 45% of $^{32}$P applied was recovered at the expected pppApPyp region, and the remainder was found in other chromatographic regions. On adding $\sigma$, $^{32}$P recovered in the pppApPyp fraction was markedly increased. The results indicate, in accordance with the previous observation with fd RF-I DNA (Sugiura *et al.*, 1970), that the $\sigma$ factor restricted initiation by RNA polymerase to specific sites on the template.

*Effect of the Salt Concentration on the Starting Sequences and Size of RNA.* The foregoing analyses were made with RNA synthesized in the presence of 0.04 M KCl. It has been shown that the RNA species transcribed on T4 DNA as template are markedly influenced by salt concentration (Qasba and Zillig, 1969; Maitra and Barash, 1969). Accordingly, RNA synthesis was carried out at 0.2 M KCl, and the synthesized products were analyzed. At such a high salt concentration, both the rate and extent of RNA synthesis on $\phi$80 DNA were markedly depressed (see Figure 3). However, the starting sequences and size of the synthesized products were essentially identical with those of RNA made at low salt concentration, except that the proportion of the smaller RNA species was considerably increased. This was in contradiction to the observation with T4 DNA, in which shorter RNA chains were rather formed at low ionic strength (Qasba and Zillig, 1969).

*The Size of RNA Synthesized in the Presence of $\rho$ Factor.* A protein factor which causes the termination of RNA chains transcribed *in vitro* on $\lambda$ DNA has been isolated and named $\rho$ factor by Roberts (1969). In order to examine the effect of the $\rho$ factor on $\phi$80 DNA-primed RNA synthesis, RNA labeled with [$\gamma$-$^{32}$P]ATP and [$^{3}$H]AMP was synthesized with and without $\rho$, and the size distribution of the synthesized products were compared. However, the $^{32}$P profiles obtained were similar with both preparations, regardless of the presence of the $\rho$ factor (Figure 8). The RNA synthesized in the presence of $\rho$ was hydrolyzed with pancreatic RNase, and the terminal fragments produced were analyzed as in the preceding section. The major fragment identified was pppApUp. It was concluded that the starting sequence and size of the RNA species starting with pppA-- was not affected by the $\rho$ factor. It was noted, in the above centrifugation analysis, that the $^{3}$H peaks at the heavier region disappeared and were found shifted to a lighter region, when

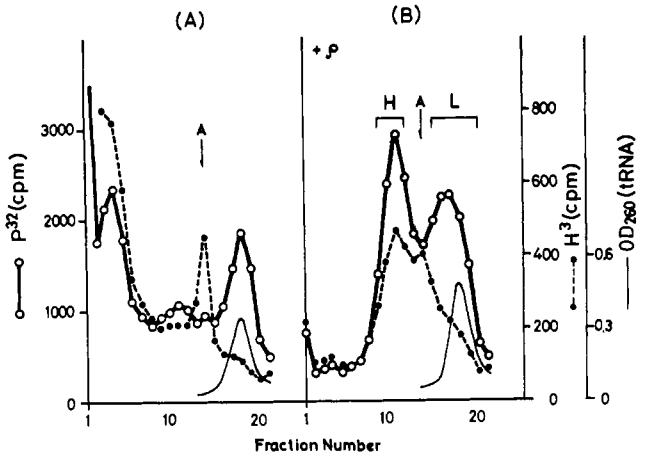


FIGURE 9: Sedimentation profiles of RNA starting with pppG–, synthesized with and without $\rho$. Two RNA-synthesizing mixtures (1 ml each) containing [$\gamma$-$^{32}$P]GTP and excess template were prepared. To one tube was added 0.2 ml of the $\rho$ fraction. At 20 min following addition of excess substrates, including [$^{3}$H]ATP, the reaction was terminated. The labeled RNA was analyzed by band centrifugation as in the text, except that the centrifugation period was for 24 hr at 25,000 rpm and 5°. *E. coli* tRNA was used as marker. (A) RNA synthesized in the absence of $\rho$ and (B) RNA synthesized in the presence of $\rho$. The arrows indicate the positions of RNA starting with pppA---.

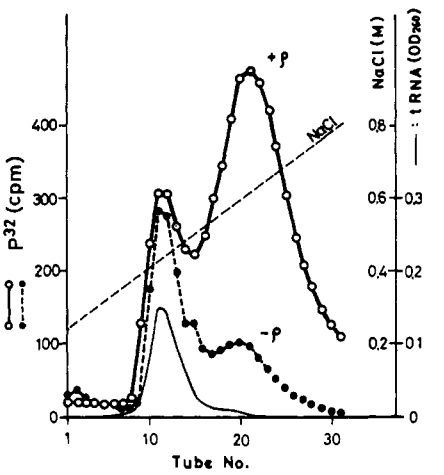


FIGURE 10: Separation of the smaller RNA components starting with pppG- -, by DEAE-cellulose column chromatography. The fraction (L) indicated in Figure 9B, which contained the RNA components less than 6–7 S was collected, charged onto a DEAE-cellulose column, and eluted with a linear gradient from 0.2 to 0.8 M NaCl (pH 8.0). As control, the corresponding region in Figure 9A was collected (tubes 14–20) and chromatographed in the similar manner. (●- - - -●) The RNA fraction from Figure 9A where RNA was synthesized in the absence of $\rho$. (○—○) The RNA fraction from Figure 9B where RNA was synthesized in the presence of $\rho$.

the $\rho$ factor had been added to the reaction mixture (Figure 8). Since the $^{3}$H peaks at the heavier region represent the RNA species starting with pppG--, it was obvious that the longer components starting with pppG-- were shortened by the action of $\rho$.

RNA labeled with [$\gamma$-$^{32}$P]GTP and [$^{3}$H]AMP was synthesized in the presence or absence of $\rho$ and analyzed by band centrifugation. The centrifugation period was increased to separate smaller RNA species, so that the 26S and 37S components synthesized without $\rho$ were sedimented to the bottom region and a $^{3}$H peak which was accounted to be the RNA species starting with pppA-- appeared in the middle of the gradient (Figure 9A). On adding $\rho$ to the reaction mixture, $^{32}$P was found distributed at two principal regions, where the S value at the peak positions were roughly 6 and 13 S, respectively. The $^{3}$H activity was also coincident with the $^{32}$P peaks (Figure 9B). In contrast to the heavier peak, however, the lighter one was relatively broad and included the marker tRNA at the front shoulder. The light half of the twin peaks shown in Figure 9B was collected, charged on a DEAE-cellulose column, and eluted with a linear NaCl gradient. As indicated in Figure 10, the $^{32}$P activity was now separated into two fractions; one eluted together with the marker tRNA and the other eluted at a relatively high salt concentration. Since the 4S component synthesized in the reaction mixture with no $\rho$ present was also eluted at the marker tRNA region on DEAE-cellulose chromatography, it was concluded that the lighter peak comprised the 4S RNA species at the front shoulder of the main peak.

RNA labeled with [$\gamma$-$^{32}$P]GTP was synthesized without $\rho$, extracted, and added to an RNA-synthesizing mixture containing $\rho$, but no radioactive substrate. The mixture was incubated for 20 min and then the RNA fraction was analyzed by band centrifugation. However, no decrease in size of $^{32}$P-labeled RNA was observed. The observation indicates that the $\rho$ factor did not break the synthesized RNA, but terminated RNA synthesis by directly interacting with the synthesizing complex (Roberts, 1969).

The light and heavy half-regions in Figure 9B were, respectively, hydrolyzed with pancreatic RNase, and $^{32}$P fragments produced were chromatographically resolved. As illustrated in Figure 11, pppGpPyp was produced predominantly

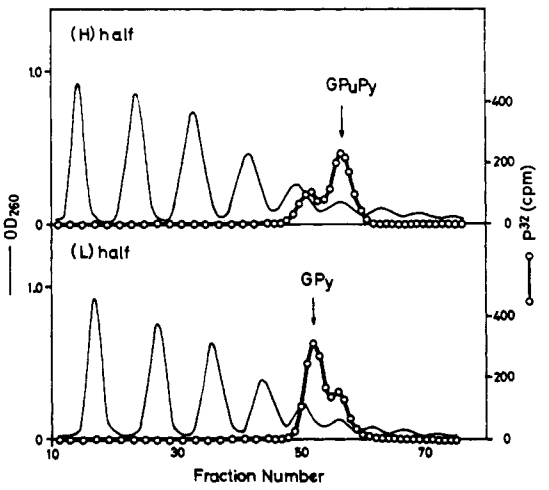


FIGURE 11: The terminal fragments produced from pancreatic RNase hydrolysates of [$\gamma$-$^{32}$P]GTP-labeled RNA synthesized in the presence of $\rho$. The (L) and (H) half-regions indicated in Figure 9B were hydrolyzed with pancreatic RNase and resulting $^{32}$P fragments were separated by DEAE-Sephadex column chromatography.

TABLE V: The Starting Sequences and Size of Major RNA Species Transcribed *in Vitro* on $\phi$80 DNA.

| Starting Sequences | Approximate *s* Values (S) | |
|---|---|---|
| | Without $\rho$ | With $\rho$ |
| pppApUpNpG----- | 10 | 10 |
| pppGpC----- | 4 | 4 |
| | 26 | 6 |
| pppGpApU------- | 13 | |
| | 26 | 13 |
| | 37 | |

from the light half and pppGpPupPyp from the heavy half. These fragments were further identified by Dowex 1 column chromatography, and it was shown that the former was exclusively pppGpCp and the latter was pppGpApUp. The 4S RNA species eluted together with the marker tRNA also produced pppGpCp by treatment with pancreatic RNase. It was concluded that the following three species of (pppG--)RNA were synthesized in the presence of $\rho$:4S RNA starting with pppGpC---, 6S RNA starting with pppGpC---, and 13S RNA starting with pppGpApU---.

## Discussion

Results of the starting sequence analyses and size determinations are summerized in Table V. When RNA was synthesized with enzyme lacking $\sigma$, the starting sequences of the synthesized products became very heterogeneous. It is evident, therefore, that RNA polymerase containing $\sigma$ selected appropriate initiation sites and transcribed fractions of the $\phi$80 DNA molecule. As reported previously (Sugiura *et al.*, 1969), we have domonstrated that three different species of RNA starting with the sequences of pppApUpG--, pppGpUpA--, and pppGpUpU-- were synthesized on RF-I DNA of phage fd. Two of these sequences are proposed initiation codons for protein synthesis (Ghosh *et al.*, 1967). In contrast to these RNA formed on fd RF-I DNA, however, none of known initiation triplets was found at the starting termini of $\phi$80 DNA-primed RNA. The nucleotide sequences at the 5′ termini of various RNA molecules from RNA-containing phages such as Q$\beta$, MS2, f2, and R17 have been analyzed (Billeter *et al.*, 1969; Bishop *et al.*, 1968; Dahlberg, 1968; Glitz, 1968; Roblin, 1968; de Wachter and Fiers, 1969). These phage RNAs exhibit a typical mRNA function in cell-free protein-synthesizing systems. However, no known initiation codon was found at or near their 5′ termini. In case of phage RNA, there is a possibility that the terminal sequences of the RNA molecules reflect the sequence requirements for the initiation of phage RNA replication.

Without the termination factor isolated by Roberts (1969), RNA starting with pppA-- gave a single peak of about 10 S, and those starting with pppG-- gave four discrete peaks of about 4, 13, 26, and 37 S. Since the band centrifugation was carried out in the presence of formamide, it is unlikely that the rapidly sedimenting components represent aggregates of shorter chains. The result appears to be essentially consistent with the observation by Cohen *et al.* (1967), who reported that four different sizes of RNA with the S values of 6–7, 12, 23, 35–45 S were synthesized *in vitro* on $\lambda$ DNA.

When Roberts' termination factor was added to the RNA-synthesizing mixture, the formation of the shorter RNA species such as the 10S RNA starting with pppA-- and 4S RNA starting with pppG-- appeared not to be influenced. However, the 26S and 37S components starting with pppG-- completely disappeared. Instead, these RNA species were found shifted to the 6S and 13S regions. The $\rho$ factor was free of endonuclease activity, as judged by the infectivity assay of f2 RNA and fd DNA on spheroplasts. The 26S and 37S components were formed only when the $\rho$ factor was not present during RNA synthesis. Since the starting sequences of the synthesized products were not influenced by the addition of $\rho$, the $\rho$ factor appears to affect only chain elongation rather than initiation of these RNA chains.

There is little information about the molecular mechanism of termination of RNA chains by the $\rho$ factor. Without the $\rho$ factor, RNA starting with the sequence of pppGpApU-- was found at the 13S, 26S, and 37S peaks (see Table III). After synthesizing RNA with $\rho$, RNA starting with pppGpApU-- was only found at the 13S peak. An interpretation of the result would be that the 26S and 37S chains were caused by incomplete termination of an RNA species which was initiated at a single initiation site. This could be due to the contamination of the $\rho$ factor in our enzyme preparation. However, unexplained is the phenomenon that such RNA species as the 10S RNA starting with pppA-- was completely terminated without adding the $\rho$ factor. As has been suggested by Roberts (1969), there could exist several factors like $\rho$ which recognize different termination signals. This being the case, one might imagine that our enzyme preparation contains some other termination factor which can terminate the 10S RNA starting with pppA--, but lacks in the factor which restricts the size of other RNA species.

As indicated in Table I, the ratio of the G termini to A termini formed was markedly increased with the enzyme concentration. Apparently, the number of respective RNA chains synthesized on $\phi$80 DNA varied with the ratio of enzyme to template used for RNA synthesis. RNA synthesis was carried out under two extreme conditions, one with excess template and the other with excess enzyme, and their starting sequences and size were compared. However, the species of RNA formed were essentially identical with both preparations. The difference was only in the quantitative ratios of the synthesized products. The observation suggests that not the selectivity of transcription but the efficiency of RNA chain initiation at a limited number of sites is influenced with the conditions used for RNA synthesis.

It has been reported that RNA synthesis reaction by RNA polymerase is highly influenced with the salt concentration. With T4 DNA as template, both the rate and extent of RNA synthesis were stimulated at high salt concentration (Qasba and Zillig, 1969; Maitra and Barash, 1969). In contrast, the $\phi$80 DNA-primed RNA synthesis was rather increased at low salt concentration. The effect of ionic strength on RNA synthesis seems to differ with the species of template.

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