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Molecular design of a eukaryotic messenger RNA and its chemical synthesis

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ABSTRACT

A designed mRNA consisting of 42 ribonucleotides having the cap structure was synthesized. The capped leader sequence of the brome mosaic virus (BMV) mRNA 4, $m^7G^{5'}$pppGUAUUAAUA (F-1), was synthesized by the phosphotriester method and followed by the capping reaction. A 32-mer consisting of an initiation codon (AUG), the coding region corresponding to a bacterial pheromone cAD1 and two stop codons, was constructed by the 18-mer (F-2) and 14-mer (F-3), which were synthesized by the phosphoramidite method. 2′-,3′-O-Methoxymethyleneguanosine 5′-phosphate was condensed with F-3 using P^1-2′,3′-O-methoxymethyleneguanosine-5′-yl P^2-adenosine-5′-yl pyrophosphate (9) with T4 RNA ligase. The chemically synthesized RNA fragments were ligated successively with T4 RNa ligase to afford the whole RNA molecule.

INTRODUCTION

Eukaryotic mRNAs have a cap structure ($m^7G^{5'}$pppNNN...) at their 5′-terminus. This structure was first found by Miura (1) in 1975 from cytoplasmic polyhedrosis virus and the structure was confirmed by its chemical synthesis (2–5). This cap structure is known to act in the important role of binding mRNAs to ribosome (6–8) and for the stabilization of mRNAs against 5′-exonucleases (9,10). 7-Methylguanosine of the cap structure is unstable under alkaline conditions. Therefore, after construction of the cap structure at the 5′-terminus of an oligoribonucleotide, protecting groups of the oligoribonucleotide should be removed under neutral or acidic conditions. We have tested several methods for the synthesis of the capped oligoribonucleotides (11–13). Consequently, the most useful method was the reaction of a 5′-phosphorimidazolide derivative of oligoribonucleotide bearing the tetrahydropyranyl(Thp) groups at the 2′-hydroxyl groups, with a capping agent, 2′,3′-O-methoxymethylene-N^2-(4,4′,4″-trimethoxytrityl)-7-methylguanosine-5′-diphosphate ($ppm^7G^{TMTr}mM$) (14,15). In this paper, we would like to report the synthesis of the capped mRNA using the chemically synthesized capped oligoribonucleotide (**F-1**).

For the synthesis of mRNA, we have first designed a man-made mRNA considering translation efficiency, easy synthesis, and stability of the mRNA as follows.

Eukaryotic mRNA generally consists of the cap structure, leader sequence, initiation codon, cistron, stop codon, 3′-noncoding region and poly A. For the leader sequence of man-made mRNA, we have chosen the leader sequence of brome mosaic virus (BMV) mRNA No.4 coding its coat protein, GUAUUAAUA. According to Yamaguchi (6), the suitable hybridization between this leader sequence and the 3′-end of 18S rRNA makes translation initiation complex effectively. A high translation efficiency of the man-made mRNA is then expected using this leader sequence. For the cistronic region, Streptococcus faecalis sex pheromone cAD1 (16) was chosen. This pheromone consists of only eight amino acids (see Figure 1). As the biological activity of this pheromone is detectable at a very low concentration of 5×10^{-11} M, it is possible to identify the formation of the peptide which would be translated from this man-made mRNA in vitro. The codon for each amino acid of this peptide was selected based on the codon usage of BMV mRNA (17). Although GUU is the most frequently used codon for valine, GUA was chosen in consideration of the ligation efficiency as described later. The initiation codon (AUG) and double stop codons (UAG) were disposed at the head and the tail, respectively, of the cistron. Contrary to the leader sequence of mRNA, little is known about the role of the 3′-noncoding region. Although it is said that this region regulates translation of mRNA (18), it will be possible to translate mRNA without the 3′-noncoding region in vitro translation system. Therefore, this region was eliminated from the man-made mRNA. The poly A tail is credited with the role for stabilization of mRNAs against 3′-exonucleases (19,20). In this experiment, the poly A tail was also eliminated to design the man-made mRNA and 2′,3′-O-methoxymethylene group was introduced to the 3′-end of the man-made mRNA for protection against 3′-exonucleases. The

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total sequence of the designed mRNA molecule is shown in Figure 1.

In order to synthesize the whole RNA molecule, it was divided into four fragments; F-1, F-2, F-3 and the 3′-terminal guanosine derivative (see Figure 1). These chemically synthesized fragments were joined by means of T4 RNA ligase from the 3′-terminus to 5′-terminus of mRNA. It is known that the nucleotide sequences surrounding the ligation position are of importance for the ligation efficiency. As a first letter of the donor oligomer (phosphate component), 5′-phosphorylated cytidine is the most suitable. On the other hand, the acceptor oligomer (hydroxyl component) containing purine rich sequence is desirable (21,22). Therefore, this fact was taken into consideration when the man-made mRNA was divided into fragments and it was the reason why GUA was chosen for the codon of valine as described above.

EXPERIMENTAL PROCEDURES

General Methods

UV spectra were obtained on a Hitachi 124 spectrophotometer. Paper chromatography was performed using a descending technique on Whatman 3MM papers with 2-propanol-conc. ammonia-water (6:1:3 v/v/v)(Solvent 1). Paper electrophoresis was performed at 1500V using Toyo Roshi No.51A paper, which was impregnated with acetic acid-morpholine buffer (pH 3.5). Two dimensional TLC was performed on precoated Avicel SF (Funakoshi) TLC plates. Anion-exchange HPLC was performed on a JASCO TRI ROTAR Type II apparatus equipped with a GP-A30 solvent programmer, a UVIDEC 100-II detector and a RC-225 recorder. Reversed phase HPLC was performed on a Waters Model-440 apparatus equipped with a Model-660 solvent programer, a M-45 pump, a column oven, and a Pantos U-228 recorder. Analysis of the digestion products by enzymatic treatment of **F-1** and **9** was performed by a μ-Bondapak C_{18} column (Waters). Pyridine was distilled twice over p-toluenesulfonyl chloride and once over calcium hydride and then stored over molecular sieves (4A). Nucleosides (Yamasa Shoyu), snake venom phosphodiesterase (Boehringer), polynucleotide kinase (3′-phosphatase free) (Boehringer), nulease P1 (Yamasa Shoyu), RNase T_2 (Seikagaku kougyo) and T4 RNA ligase (Pharmacia) were purchased. Kination buffer was prepared by mixing 5 μL of 1 M Tris-HCl (pH 8), 2 μL of 0.5 M $MgCl_2$, 5 μL of 0.04 M spermidine, 10 μL of 0.1 M dithiothreitol and 8 μL of sterilized water. Ligation buffer was prepared by mixing 0.9 mL of 0.5 M HEPES-NaOH (pH 8.3), 0.9 mL of 0.1 M dithiothreitol, 0.9 mL of 0.1 M $MgCl_2$, 45 μL of 2 mg mL^{-1} bovine serum albumin and 1.76 mL of sterilized water. RNA sequencing was performed by the method of Donis-Keller (26,27) and Boguski (28). The 5′-[^{32}P] labeled RNA fragment was treated with RNase T_1 (G specific) (Seikagaku Kougyo), RNase U_2 (G and A specific) (Seikagaku Kougyo), RNase Phy M (U and A specific) (Pharmacia) and chicken liver RNase (C specific) (Pharmacia) under partial digestion conditions, and the products were detected by 20% polyacrylamide gel electrophoresis.

Synthesis of the 2′-Thp-Protected 9-mer (2)

To a solution of **1** (62.6 mg, 7.18 μmol) in pyridine-water (4.6:1 v/v, 2.8 mL) was added silver acetate (599 mg, 3.59 mmol). The mixture was stirred at 50°C for 5 h. After cooling, it was diluted with pyridine-water (4:1 v/v, 10 mL). Removal of the silver ion was performed by the procedure descrived previously (15). The residue was dissolved in pyridine-conc. ammonia (3:5 v/v, 80 mL) and it was kept in a sealed flask at 50°C for 21 h. Further, pyridine-conc. ammonia (2:5 v/v, 45 mL) was added and kept at 50°C for 7 h. The solution was diluted with pyridine (60 mL) and concentrated under reduced pressure. The residue was dissolved in water and washed with ether. The aqueous layer was collected and concentrated in the presence of a two-fold volume of pyridine to avoid loss of the Thp group. It was chromatographed on Whatman 3MM papers (Solvent 1). The slowest moving band was eluted with water to give the ammonium salt of **2** (476 OD, 72%, UV λ max 259 nm, and λ min 230 nm).

Synthesis of the 5′-Terminal Phosphorimidazolide Derivative of 2 (3)

Compound **2** (426 OD, ammonium salt, 4.6 μmol) was converted to the trioctylammoium salt as described previously (15). The residue was coevaporated five times with dry pyridine and dissolved in dry DMF (1.2 mL), then carbonyldiimidazole (15 mg, 93 μmol) was added. After being stirred for 3 h, carbonyldiimidazole (7.5 mg, 46 μmol) was further added with continuous stirring for 1 h. The reaction was quenched by the addition of methanol (9 μl, 222 μmol). After being stirred for 30 min, the mixture was concentrated under reduced pressure and the residue was applied to the capping reaction without further purification.

The Capping Reaction of 3 with the agent 4

Compound **4** (23 μmol) was rendered anhydrous by repeated coevaporation with dry pyridine and dissolved in dry DMF (1 mL), then added to compound **3** (4.6 μmol). After being stirred at room temperature for 48 h, the reaction was quenched by the addition of water and the mixture was concentrated under reduced pressure. The residue was coevaporated several times with toluene-methanol (1:1 v/v), and a solution of 0.01 M HCl in dioxane-water (1:1 v/v, 17 mL) was added. Dioxane was further added to make it homogeneous and then adjusted to pH 2.0 by the addition of 0.1 M HCl. After being stirred for 26 h at room temperature, it was neutralized with 1 M aqueous ammonia and concentrated under reduced pressure to remove dioxane. The aqueous solution was washed with ether and concentrated under reduced pressure. The residue was dissolved in sterilized water (10 mL) and applied to a column of DEAE Sephadex A-25 (1.5×30 cm). Elution was successively performed with sterilized water (100 mL), 0.3 M NH_4HCO_3 (200 mL), and 1 M NH_4HCO_3 (100 mL). Fractions of the UV perceived region were collected during the elution with 1 M NH_4HCO_3. They were combined and concentrated under reduced pressure. The residue was coevaporated several times with sterilized water to give crude **5**. An aliquot of the crude **5** (3.8 μmol) was purified by anion-exchange HPLC using a Whatman Partisil 10-SAX

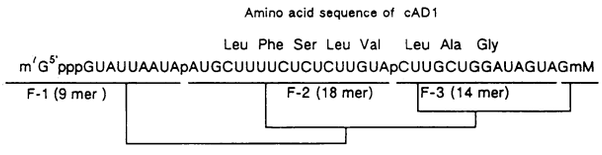


Figure 1. Design of a Man-made mRNA by the Fragment Condensation Using T4 RNA Ligase.

column. A peak eluted at 36 min was collected and desalted with DEAE Sephadex A-25 in the usual manner. Compound **5** was then obtained (21 OD, overall yield from **1** was 5.4%, λ max. 259 nm, λ min. 230 nm).

Enzymatic Assay of 5

Freeze-dried **5** (1 OD) was incubated with nuclease P1 (2 mg mL^{-1}, 10 μL) in 0.05 M sodium acetate buffer (pH 5.4, 90 μL) at 37°C for 5 h. Reversed phase HPLC showed a mixture of $m^7G^{5'}pppG$, pU, pA in the ratio of 1.02:4.58:3.46.

Synthesis of 9

2',3'-O-Methoxymethylene guanosine 5'-phosphate(**7**) (12) (200 mg, 0.49 mmol) was coevaporated with pyridine and water in the presence of tributylamine (118 μL, 0.49 mmol). The residue was further coevaporated with dry pyridine several times and dissolved in dry DMF (6 mL). Morpholine (1 mL, 11.5 mmol), triphenylphosphine (259 mg, 0.98 mmol) and 2,2'-dipyridyl disulfide (PySSPy) (228 mg, 0.98 mmol) were successively added and stirred at room temperature for 3 h. After addition of water (0.5 mL), the mixture was concentrated and the residue was partitioned into ether (20 mL) and water (20 mL). The aqueous layer was washed with ether (20 mL×4) and concentrated to a gummy residue. It was dissolved in CH_3OH and the precipitate appeared by the addition of ether. The supernatant was decanted and precipitate was collected and dried. It was applied to paper chromatography (Solvent 1) to give 2',3'-O-methoxymethylene-guanosine 5'-phosphormorpholidate (**8**) (206 mg, 84%). Compound **8** (19 μmol) was mixed with the monooctylammonium salt of 5'-AMP (29 μmol) and dissolved in dry DMF. After being stirred at 50°C for 20 h, the mixture was concentrated and a part of the residue (0.5 μmol) was purified by paper electrophoresis to give **9** (5.3 OD, 39%, UV λ max 255 nm, λ min 225 nm).

Deprotection and Enzymatic Assay of 9

Two diastereoisomers of **9** were separated by reversed phase HPLC. Each diastereoisomer (0.55 OD for 13.5 min, 2.1 OD for 14 min) was dissolved in 80% acetic acid (1 mL). After being stirred for 16 h, it was concentrated, then purified by reversed phase HPLC. $A^{5'}ppG$ was obtained in 0.27 OD from the former and 1.36 OD from the latter. Each product (0.27 OD for former and 0.5 OD for latter) was incubated with snake venom phosphodiesterase (1 mg mL^{-1}, 4 μL) in the presence of 1 M $MgCl_2$ (2 μL) in Tris-HCl buffer (pH 8.0, 40 μL) at 37°C for 3 h. Reversed-phase HPLC showed a mixture of pG and pA in a ratio of 1:1.

Synthesis of 15-mer

F-3 (0.149 OD, 1.1 nmol) and **9** (0.354 OD, 14.4 nmol) were dissolved in a ligation buffer (16 μL) and diluted with sterilized water (13 μL). To the solution, T4 RNA ligase (33 unit, 4 μL) was added. After incubation at 20°C for 1.5 h, it was allowed to stand at 4°C for 12 h. The mixture was heated at 80°C for 2 min and immediately cooled in an ice bath. A part of this solution (30 μL) was mixed with [γ-^{32}P]ATP (4μL, 10 μCi/μL) and polynucleotide kinase (1 μL, 10 u/μL). It was incubated at 37°C for 45 min, and further treated with ATP (11 μL, 1 mM) in the presence of polynucleotide kinase (1 μL, 10 u/μL) at 37°C for 2 h. The mixture was then heated at 80°C for 2 min. To the solution, dye-formamide (5:45 v/v, 7 μL) was added and then subjected to 20% polyacrylamide gel electrophoresis. After autoradiography, the desirable 15-mer was extracted from the gel by 0.5 M sodium acetate at 37°C for 12 h. The supernatant was purified by C_{18} reversed phase column chromatography to give 15-mer (0.49 nmol, 49%).

Synthesis of 33-mer

The 15-mer (492 pmol) was mixed with a trace amount of the freshly purified 5'-[^{32}P] labeled 15-mer to increase the intensity of the autoradiogram. To this component was added **F-2** (0.23 OD, 1.48 nmol), ATP (2.1 μL, 10 mM) and T4 RNA ligase (10 μL, 8 u/μL) in ligation buffer (9 μL). After incubation at 4°C for 22 h, the mixture was heated at 80°C for 2 min and cooled in ice bath. The mixture was applied to 12% polyacrylamide gel electrophoresis and followed by a procedure similar to that described previously. The 33-mer was then obtained (197 pmol, 40%).

5'-Phosphorylation of 33-mer

The 33-mer (157 pmol) was dissolved in sterilized water (2 μL) and treated with a mixture of ATP (2 μL, 10 mM) and polynucleotide kinase (4 μL, 10 u/μL) in HEPES buffer (5 μL). In a similar manner, a small portion of the 33-mer (5 pmol) was treated with [γ-^{32}P]ATP (2 μL, 10 μCi/μL) and polynucleotide kinase (2 μL, 10 u/μL) in kination buffer (5 μL). Each solution was incubated at 37°C for 3 h and heated at 80°C for 2 min and then cooled in an ice bath. Both the 5'-[^{31}P] 33-mer and 5'-[^{32}P] 33-mer were combined and purified by means of 12% polyacrylamide gel electrophoresis. They were extracted from the gel by 0.5 M sodium acetate at 37°C for 12 h. The supernatant was purified on a Sephadex G-25 column using 0.05 M ammonium acetate to give the 5'-phosphorylated 33-mer (94 pmol, 60%).

Synthesis of Capped 42-mer

The 5'-phosphorylated 33-mer (94 pmol) was dissolved in a mixture of **F-1** (0.26 OD, 2.5 nmol) and ATP (2.5 μL, 10 mM) in a ligation buffer (11 μL), then T4 RNA ligase (11.5 μL, 14.1 u/μL) was added. The mixture was incubated at 20°C for 1 h, then at 4°C for 12 h. After heating at 80°C for 2 min, it was applied to 12% polyacrylamide gel electrophoresis and followed

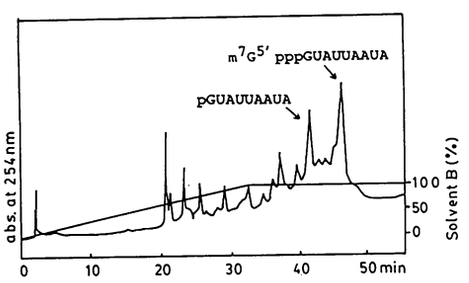


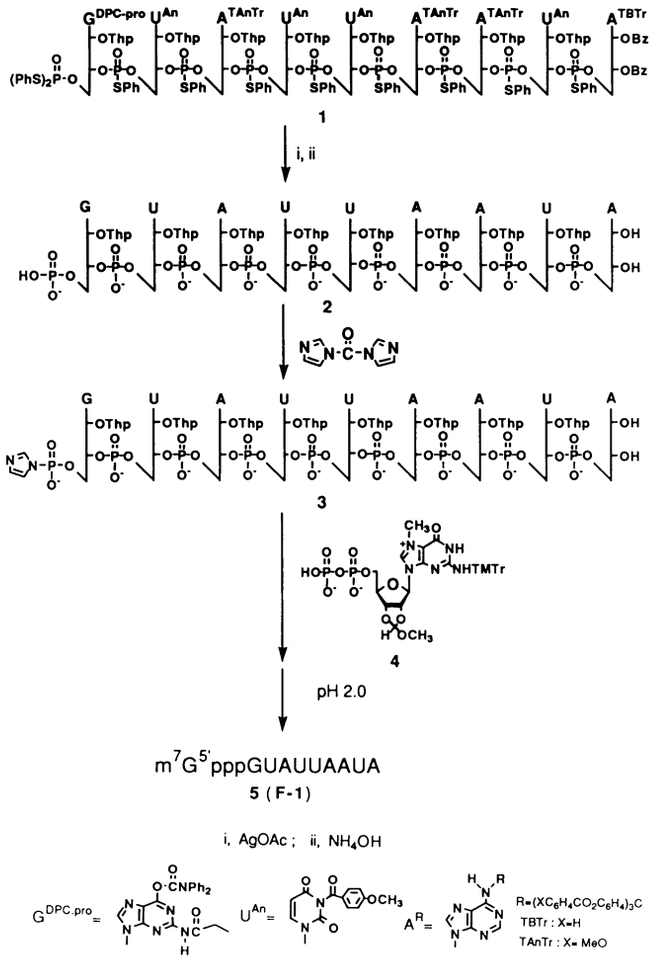
Figure 2. Anion-exchange HPLC of Crude $m^7G^{5'}pppGUAUUAAUA$ Obtained After Chromatography Using a Sephadex A-25 Column. The following solvent system was used; a linear gradient(0−99%) starting from buffer A (0.005 M KH_2PO_4, 20% CH_3CN, pH 4.1) to buffer B (0.5 M KH_2PO_4, 20% CH_3CN, pH 4.5) at the flow rate of 1.5 mL min^{-1} for 32 min.

Table 1. Ligation of Oligoribonucleotides with T4 RNA Ligase.

acceptor (nmol)	donor (nmol)	ATP (mM)	T4 RNA ligase (u/μL)	temp. (℃)	time (h)	reaction yield (%)	isolated yield (%)
F-3 (1.1)	$A^{5'}$ppGmM (14.4)	—	1	20 then 4	1.5 then 12	86[a]	49[a]
F-2 (1.5)	pCUUGCUGGAUAGUAGmM (0.49)	1	3.8	4	22	78	40
$m^7G^{5'}$pppGUAUUAAUA (2.5)	pAUGCUUUUCUCUCUUGUACUUGCUG-GAUAGUAGmM (0.09)	1	6.5	20 then 4	1 then 12	90	58

[a] Yield indicates the 5'-phosphorylated 15-mer.

Scheme I



by a work up similar to that previously described to give the capped 42-mer (54 pmol, 58%).

Sequence Analysis of the Capped 42-mer

The capped 42-mer (1.5 pmol) was dissolved in a solution (4 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM mercaptoethanol and 1 mM spermidine, and followed by

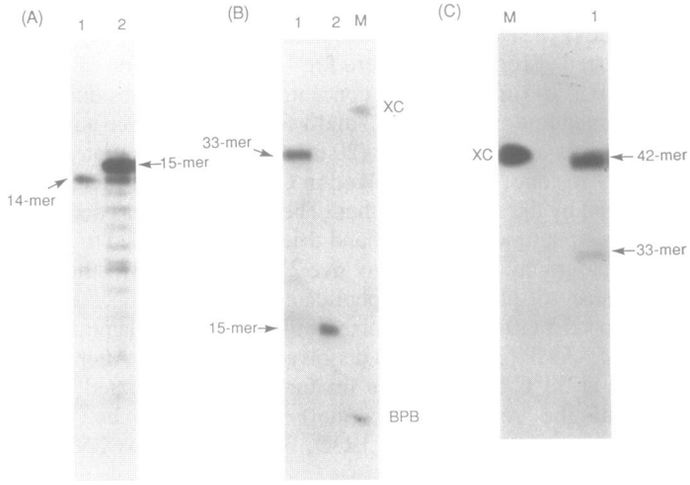
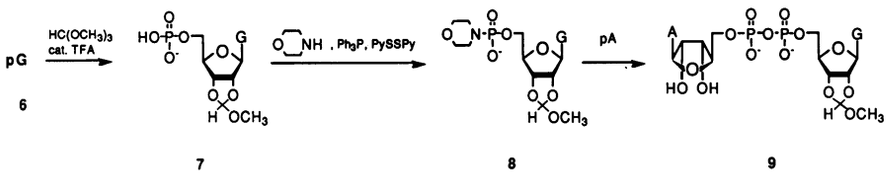


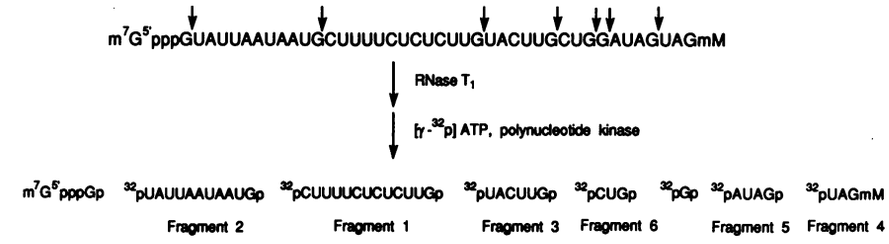
Figure 3. Polyacrylamide Gel Electrophoresis of the Ligated Products. (A) 15-mer from **F-3** (14-mer) and $A^{5'}$ppGmM. Lane 1, 5'-phosphorylated **F-3** (14-mer); Lane 2, ligated product. (B) 33-mer from **F-2** (18-mer) and 5'-phosphorylated 15-mer. Lane 1, ligated product; Lane 2, 5'-phosphorylated 15-mer; Lane M, markers. (C) capped 42-mer obtained by ligation of **F-1** and 5'-phosphorylated 33-mer. Lane 1, ligated product; Lane M, markers.

the addition of RNase T_1 (1 μL, 1 u/μL). After incubation at 37°C for 1 h, [γ-^{32}P]ATP (3 μL, 10 μCi/μL) and polynucleotide kinase (1 μL, 10 u/μL) were added. After incubation at 37°C for 1 h, formamide (5 μL) was added and applied to 20% polyacrylamide gel electrophoresis. After autoradiography, each product (Fragment 1−6, see Figure 4) was harvested and extracted by 0.5 M sodium acetate. Ethanol was added to each supernatant and a precipitate appeared. It was collected and subjected to the analysis of 5'-terminal nucleotide as follows: A part of each precipitate (ca. 15000−20000 cpm) was treated with nuclease P1 (1 μL, 1 mg mL^{-1}) in 5 μL of 40 mM NH_4OAc (pH 5) at 37°C for 2 h. The resulting solution was subjected to two dimensional cellulose TLC: The developing solvents for the 1st and 2nd dimensions were isobutyric acid−0.05 M aqueous ammonia (5:3 v/v) and 0.1 M sodium phosphate (pH 6.8)−ammonium sulfate−2-propanol (100:60:2 v/w/v), respectively. The 5'-[^{32}P] labeled nucleotide was identified by comparison with an authentic nucleoside 5'-phosphate.

Scheme II



Scheme III



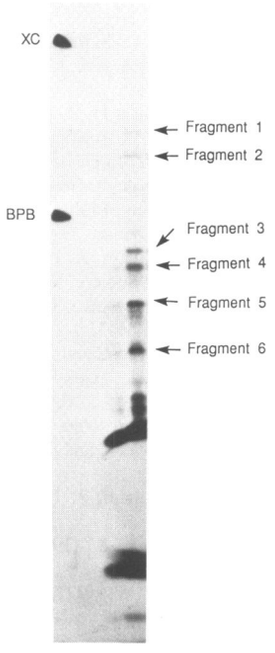


Figure 4. Polyacrylamide Gel Electrophoresis of Digested 42-mer with RNase T_1.

RESULTS AND DISCUSSION

First, the capped leader sequence, $m^7G^{5'}pppGUAUUAAUA$, was synthesized according to the phosphotriester method (23) followed by the capping reaction (14,15). A fully protected RNA fragment of the leader sequence (**1**) was converted to the 2'-Thp derivative of 9-mer having 5'-phosphate (**2**) by treatment with silver acetate followed by conc. ammonia as shown in Scheme I. For the construction of the cap structure at its 5'-terminus, it was converted into the phosphorimidazolide (**3**) and reacted with the capping agent ($ppm^7G^{TMTr}mM$) (**4**). Finally, whole protecting groups were removed under acidic conditions to afford the capped 9-mer, $m^7G^{5'}pppGUAUUAAUA$ (**5**, **F-1**). Purification of **F-1** was performed by means of DEAE Sephadex A-25 column chromatography and anion exchange HPLC as shown in Figure 2. The structure was confirmed by enzymatic digestion using nuclease P1.

Next, two RNA fragments, 18-mer (**F-2**) and 14-mer (**F-3**) (see Figure 1) were synthesized according to the phosphoramidite method previously reported (24,25). The sequence of the fragments was analyzed by the method of Donis-Keller (26,27) and Boguski (28). The 3'-terminal nucleotide of these fragments was confirmed by RNase T_2 digestion after the ligation of ^{32}pCp at 3'-terminus of these fragments.

It is known that the ligation of RNA requires the protection of 3'-terminal hydroxyl group of donor molecule (phosphate component) to avoid self-oligomerization or cyclization (29,30). Among several protecting groups (31−39), the methoxymethylene group was chosen and introduced to 2'- and 3'-hydroxyl groups of 3'-terminal guanosine of the mRNA (see Figure 1) for the ligation with **F-3**. Namely, guanosine 5'-phosphate (**6**) was converted to 2',3'-O-methoxymethyleneguanosine 5'-phosphate (**7**) (Scheme II) (12). However, it is reported that the adenylated donor molecule ($A^{5'}ppN$) is an effective substrate to produce the ligation because it is an activated intermediate (32,35,40−42). Therefore, **7** was converted to P^1-2',3'-O-methoxymetyleneguanosine-5'-yl P^2-adenosine-5'-yl pyrophosphate (**9**) by a modification of the procedure of Moffatt (43). First, **7** was transformed into the phosphormorpholidate (**8**) by the Mukaiyama condensation (44) and it was then allowed to react with AMP to give **9**. Compound **9** was isolated by paper electrophoresis and the HPLC profile showed two peaks due to the chirality of the methoxymethylene group. It was proved by deprotection and enzymatic digestion.

The ligation of RNA fragments (see Figure 1) was performed in the direction from 3'- to 5'-fragments as follows; first, **9** was ligated efficiently with **F-3** (14-mer) using T4 RNA ligase. After kination of the 15-mer using ATP with polynucleotide kinase,

it was isolated by polyacrylamide gel electrophoresis. It was successively ligated with **F-2** (18-mer) to obtain the 33-mer. The Donnis-Keller method was used for the sequencing of the 33-mer.

In a similar manner, the 33-mer was kinated and further ligated with **F-1** to give the desired 42-mer having the cap structure in 58% yield. The reaction conditions and the results are shown in Table I and Figure 3.

Efficiency of the ligation was found to depend on the amount of T4 RNA ligase. For the ligation of the F-2 (18-mer) with the 15-mer, the reaction yield increased from 4% to 78% as increasing mount of T4 RNA ligase from 1.6 u/μL to 3.8 u/μL. Similarly, yield of the capped 42-mer was improved from 55% to 90%.

The 42-mer was confirmed by enzymatic digestion with RNase T_1 similar to that reported by Tanaka (see Scheme III) (45). First, it was degraded to the short oligomers as pointed out by the arrow in Scheme III. The reaction mixture was then labeled by [γ-^{32}P]ATP with polynucleotide kinase, and the labeled oligomers were separated by polyacrylamide gel electrophoresis as shown in Figure 4. The six spots corresponding to the pCUUUUCUCUCUUGp, pUAUUAAUAAUGp, pUACUUGp, pUAGmM, pAUAGp and pCUGp were detected, although the spot of pGp could not be detected because the estimated position was covered with [γ-^{32}P]ATP. Each fragment was isolated and digested by nuclease P1 and the 5'-terminal nucleotide of the oligomer was confirmed by two dimensional TLC.

It seemed that the capped 9-mer (**F-1**) could possibly react with the 3'-hydroxyl group of 7-methylguanosine during ligation. However, Kohno (46) found that 3'-hydroxyl group of 7-methylguanosine did not act as an acceptor for ligation of the shorter oligomer using T4 RNA ligase. Actually in this experiment, the 3'-hydroxyl group of 7-methylguanosine of **F-1** did not react with the 33-mer and the desired capped 42-mer was obtained.

In conclusion, the man-made mRNA having the cap structure could be synthesized by combination of the chemical synthesis of RNA fragments and their ligation using T4 RNA ligase. More recently, the RNAs can be obtained enzymatically using T7 RNA polymerase or SP6 RNA polymerase (47−51). However, the resulting RNAs contain 5'-proximal sequences of the vector sequence or there exists the limitation of the sequence accuracy such as one nucleotide shorter sequence than that expected. On the contrary, the chemical method described here enabled us to correctly synthesize the desirable capped mRNAs.

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