



HAL
open science

High-yield production of short GpppA- and 7MeGpppA-capped RNAs and HPLC-monitoring of methyltransfer reactions at the guanine-N7 and adenosine-2'-O positions.

F. Peyrane, B. Selisko, Etienne Decroly, Jean-Jacques Vasseur, D. Benarroch, B. Canard, Karine Alvarez

► To cite this version:

F. Peyrane, B. Selisko, Etienne Decroly, Jean-Jacques Vasseur, D. Benarroch, et al.. High-yield production of short GpppA- and 7MeGpppA-capped RNAs and HPLC-monitoring of methyltransfer reactions at the guanine-N7 and adenosine-2'-O positions.. *Nucleic Acids Research*, 2007, 35 (4), pp.1-11. 10.1093/nar/gkl1119 . hal-00139133

HAL Id: hal-00139133

<https://hal.science/hal-00139133>

Submitted on 1 Jun 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

High-yield production of short GpppA- and ⁷MeGpppA-capped RNAs and HPLC-monitoring of methyltransfer reactions at the guanine-N7 and adenosine-2'O positions

F. Peyrane¹, B. Selisko¹, E. Decroly¹, J. J. Vasseur², D. Benarroch¹, B. Canard¹ and K. Alvarez^{1,*}

¹Centre National de la Recherche Scientifique and Universités d'Aix-Marseille I et II, UMR 6098, Architecture et Fonction des Macromolécules Biologiques, AFMB-CNRS-ESIL, Case 925, 163 avenue de Luminy, 13288 Marseille Cedex 9, France and ²LCOBS, UMR 5625 CNRS-UMII, CC 008, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

Received August 31, 2006; Revised October 27, 2006; Accepted December 7, 2006

ABSTRACT

Many eukaryotic and viral mRNAs, in which the first transcribed nucleotide is an adenosine, are decorated with a cap-1 structure, ⁷MeG^{5'}-ppp^{5'}-A_{2'}OMe. The positive-sense RNA genomes of flaviviruses (Dengue, West Nile virus) for example show strict conservation of the adenosine. We set out to produce GpppA- and ⁷MeGpppA-capped RNA oligonucleotides for non-radioactive mRNA cap methyltransferase assays and, in perspective, for studies of enzyme specificity in relation to substrate length as well as for co-crystallization studies. This study reports the use of a bacteriophage T7 DNA primase fragment to synthesize GpppAC_n and ⁷MeGpppAC_n (1 ≤ n ≤ 9) in a one-step enzymatic reaction, followed by direct on-line cleaning HPLC purification. Optimization studies show that yields could be modulated by DNA template, enzyme and substrate concentration adjustments and longer reaction times. Large-scale synthesis rendered pure (in average 99%) products (1 ≤ n ≤ 7) in quantities of up to 100 nmol starting from 200 nmol cap analog. The capped RNA oligonucleotides were efficient substrates of Dengue virus (nucleoside-2'-O)-methyltransferase, and human (guanine-N7)-methyltransferase. Methyltransfer reactions were monitored by a non-radioactive, quantitative HPLC assay. Additionally, the produced

capped RNAs may serve in biochemical, inhibition and structural studies involving a variety of eukaryotic and viral methyltransferases and guanylyltransferases.

INTRODUCTION

The cap is a unique structure at the 5'-end of viral and cellular eukaryotic mRNAs (1,2). It is critical for both mRNA stability and binding to the ribosome during translation. Cap 0 (⁷MeG^{5'}-ppp^{5'}-N) formation is a co-transcriptional modification resulting from three enzymatic activities: RNA triphosphatase, guanylyltransferase and S-adenosyl-L-methionine (AdoMet) dependent (guanine-N7)-methyltransferase (N7MTase). Further methylation at the 2'O position of the ribose of the first nucleotide by an AdoMet-dependent (nucleoside-2'-O)-methyltransferase (2'OMTase) leads to a cap 1 structure (⁷MeG^{5'}-ppp^{5'}-N_{2'}OMe). Capped RNA molecules are required as substrates for the biochemical characterization of mRNA cap MTases, as well as structural studies on guanylyltransferases and MTases complexed with products or substrates, and they are not commercially available.

The single-stranded positive-sense RNA genome of flaviviruses is decorated with a cap 1 structure. The genus *Flavivirus* contains at least 70 viruses (3), among them emerging human pathogens such as Dengue, West Nile and Japanese encephalitis virus (4), all of which bear a ⁷MeG^{5'}-ppp^{5'}-A_{2'}OMe cap structure with a strictly conserved adenosine as first nucleotide. We set out to

*To whom the correspondence should be addressed. Tel: +33 491 828630; Fax: +33 491 828646; Email: Karine.Alvarez@afmb.univ-mrs.fr
Present address:

D. Benarroch, Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

produce ($^{7\text{Me}}$)GpppA-capped (i.e. GpppA- or $^{7\text{Me}}$ GpppA-) RNA molecules to aid our studies on the capping machinery of flaviviruses (5,6).

In order to get GpppA-capped RNAs, several approaches can be taken that differ widely in their efficiency. They can be synthesized chemically starting from mono- or diphosphate RNA (7,8). A $^{7\text{Me}}$ GpppA cap can also be added to di- or triphosphate RNA using vaccinia virus capping enzyme that contains RNA triphosphatase, guanylyltransferase and N7MTase activities (9,10). However, diphosphate and triphosphate RNAs are not commercially available. ($^{7\text{Me}}$)GpppA can be incorporated upon *in vitro* transcription by bacteriophage T7, T3 or SP6 DNA-dependent RNA polymerases (DdRp) (e.g. (11)) using their respective promoters and an adenosine in position +1 or +2 (12,13). The efficiency of these reactions is rather low because these promoters require a guanine as starting nucleotide. Very recently, an alternative, the bacteriophage T7 class II ϕ 2.5 promoter (14) which requires an adenosine as first transcribed nucleotide, was successfully applied to generate GpppA-capped transcripts (15). ($^{7\text{Me}}$)GpppA was reported to be incorporated upon *in vitro* transcription by *E. coli* DdRp using the bacteriophage λ PL promoter (16), a method that has not been exploited since. Finally, very short capped oligonucleotides ($^{7\text{Me}}$)GpppACC were produced efficiently using bacteriophage T7 DNA primase-helicase (17).

Here we report the adaptation of the latter approach to prepare large amounts of capped RNA oligonucleotides $^{7\text{Me}}$ GpppAC_{*n*} and GpppAC_{*n*} with a range of chain lengths ($1 \leq n \leq 7$). Instead of the full-length bacteriophage T7 DNA primase-helicase, we used a recombinant N-terminal fragment of 30 kD bearing the primase activity (18,19). The use of the fragment offers several advantages regarding enzyme production and purification compared to the full-length protein namely a 10 to 20 times higher relative expression yield, higher solubility and the fact that it does not co-purify with DNA (19). The DNA primase fragment was reported to show similar activity to the full-length protein when using high DNA template concentrations (19). Nevertheless, production of short-capped RNA oligonucleotides $^{7\text{Me}}$ GpppAC₂ that has been shown for full-length protein (17), has never been demonstrated for the DNA primase fragment. In this work, we synthesized $^{7\text{Me}}$ GpppAC₂ and GpppAC₂ as well as longer products using the DNA primase fragment. After undertaking optimization studies, we designed a robust and effective method to produce high amounts of pure ($^{7\text{Me}}$)GpppAC_{*n*} ($1 \leq n \leq 7$). Given the lack of commercial availability of capped RNA oligonucleotides we expect this method to be useful for many researchers working in the still emerging field of characterization of viral and mammalian RNA capping machineries. In addition, we demonstrate that the produced GpppA-capped RNAs are substrates of Dengue virus 2'OMTase and human mRNA cap N7MTase. Both methyltransferase reactions were monitored by a non-radioactive, quantitative assay based on HPLC and the reaction products were characterized.

MATERIAL AND METHODS

Protein expression and purification

T7 DNA primase fragment. The T7 DNA primase expression vector pET19b/PrD was kindly provided by Charles C. Richardson (Harvard Medical School, Boston, USA). *E. coli* BL21(DE3) cells (500 ml) transformed with pET19b/PrDT were grown in Luria-Bertani medium containing ampicillin. At an OD₆₀₀ of 0.6 isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μ M, and expression was allowed to proceed for 2 h at 37°C. The cellular pellet was resuspended in 10 ml lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with 1 mM benzamidine, 10 mM imidazole, 100 μ g/ml lysozyme and 1 μ g/ml DNase I. Since the expressed protein bears a His-tag at the N-terminus, immobilized-metal-affinity chromatography (IMAC) was used for the first purification step (chelating sepharose fast flow resin (Amersham Biosciences) loaded with Ni²⁺). The protein was eluted with lysis buffer containing 250 mM imidazole. Fractions were then directly loaded onto a HiTrap DEAE Sepharose FF 1-ml column (Amersham Biosciences) equilibrated with 50 mM HEPES, pH 7.5, containing 0.1 mM DTT, and eluted with a gradient of NaCl (0–500 mM). The protein elutes at 200 mM NaCl. The sample was diluted to lower the NaCl concentration to 50 mM and loaded onto a HiTrap Blue Sepharose 1-ml column (Amersham Biosciences) equilibrated with 50 mM HEPES, pH 7.5, containing 1 mM DTT and 1 mM EDTA. Elution was carried out with a gradient of NaCl (0–800 mM). Glycerol was added to reach 50% and the protein stored at –20°C.

Dengue virus MTase domain (NS5MTase_{DV}). Recombinant NS5MTase_{DV} was produced as described in (6).

Human MTase (hMTase). The cDNA coding for hMTase was a kind gift from Aaron J. Shatkin (Center of Advanced Biotechnology and Medicine, Piscataway, USA). It was amplified by PCR using primers RBSATGLys6HishMTs 5'-CCATGAAACATCACCAT CACCATCACGCAAATTCTGCAAAAAGCAGAAG-3' and hMTstopattb2as 5'-GGGGACCACTTTGTACAAG AAAGCTGGGTCTTACTGCTGTTTCTCAAAGGCA AAC-3'. A second PCR was then used to add the attb1 recombination site to the 5' using primers attB1RBS ATGLys6His 5'-GGGGACAAGTTTGTACAAAAAAG CAGGCTTCTAAGGAGGTAGAACCATGAAACAT CACCATCACCATCAC-3' and hMTstopattb2as given above. The PCR product was then cloned into the expression vector pDest14 (Invitrogen) using the Gateway technology. *E. coli* Rosetta(DE3) cells were transformed with pDest14/6His-hMTase and grown in Luria-Bertani medium containing ampicillin and chloramphenicol. At an OD₆₀₀ of 0.6, IPTG and ethanol were added to a final concentration of 100 μ M and 2%, respectively, and expression was allowed to proceed for 18 h at 17°C. The cellular pellet was resuspended in 10 ml lysis buffer (50 mM Tris, pH 8.5, 300 mM NaCl,

10% glycerol, 5 mM β -mercaptoethanol, antiprotease cocktail (Complete[®], Roche) supplemented with 10 mM imidazole, 100 μ g/ml lysozyme and 1 μ g/ml DNase I and 0.5% triton X100. After lysis by sonication and clarification, immobilized-metal-affinity chromatography (IMAC) was used for the first purification step (chelating sepharose fast flow resin (Amersham Biosciences) loaded with Ni²⁺). The hMTase was eluted with lysis buffer pH 7.5 containing 250 mM imidazole. Fractions were then diluted 5 times in 50 mM Bis-Tris pH 6.8; 50 mM NaCl, 10% glycerol, 5 mM β -mercaptoethanol, and loaded onto a 5-ml heparine column (Amersham Biosciences). The hMTase was then eluted with a gradient of NaCl (0–1 M) using a buffer at pH 7.5. The protein elutes at between 150 and 350 mM NaCl. It was finally dialyzed against 50 mM Tris, pH 7.5, 300 mM NaCl, 50% glycerol, 5 mM β -mercaptoethanol and stored at -20°C .

HPLC set-up

A Waters model 600 gradient HPLC system equipped with two 600 pumps, a 717 plus Autosampler injector, a 996 photodiode array detector and an in-line degasser AF was employed for reverse-phase chromatography. The column assembly consisting of a pre-column (Delta-pak C18 100 Å, 5 μ m, 3.9 \times 20 mm) and the separation column (Nova-pak C18, 4 μ m, 3.9 \times 150 mm) was protected by a filter insert. For the on-line cleaning procedure, both columns were installed in parallel on a two 7000 Rheodyne valve system (Interchim). As buffer stock solution, a 1 M solution of triethylammonium bicarbonate (TEAB) was prepared by adding dry-ice to a 1 M triethylamine solution until the pH reached 7.4 and filtered through 0.22 μ m GV-type membranes (Millipore). HPLC eluents were freshly prepared. Eluent A was a 0.05 M solution of TEAB (pH 7.4) and eluent B was a 1:1, v/v, mixture of acetonitrile (HPLC grade, SDS) and TEAB (final concentration 0.05 M, pH 7.4). Separations were run at a flow rate of 1 ml/min and started with a 5-min elution (100% eluent A) on the pre-column to remove proteic material. The applied gradients for analytical and preparative separation of capped RNAs as well as for analysis of methyltransferase reactions are explained in the corresponding sections (see below).

Mass spectrometry analysis

MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (PerSeptive Biosystems) equipped with an N₂ laser (337 nm). MALDI conditions were: accelerating potential, 24 000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns. Spectra were obtained in negative mode and were not smoothed. The oligonucleotides (100 pmol) were suspended in 10 μ l of water and desalted using drop dialysis through a membrane filter WSWP 0.025 μ m, 13 mm (Millipore) floating on a 0.1 M ammonium citrate solution for 30 min. When drop dialysis did not allow to remove totally salt traces, the samples were further treated with a few beads of DOWEX 50 W X8 resin (ammonium form)

before spotting them on the MALDI target. Samples of 0.5 μ l were mixed with 0.5 μ l of the matrix 2,4,6-trihydroxyacetophenone (THAP, 45 mg, ammonium citrate, 4 mg in 500 μ l acetonitrile/water, 1:1, v/v) and the mixtures were spotted on the stainless steel MALDI target and left to dry under air before MALDI analysis.

Capped RNA synthesis

Analytical scale. Experiments were carried out in 20–40 μ l reaction volume containing T7 DNA primase reaction buffer (40 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM potassium glutamate, 1 μ M ZnCl₂), 10 mM DTT and 50 μ g/ml BSA as well as CTP (Amersham Biosciences), cap analog (New England Biolabs), DNA oligonucleotides (Invitrogen, HPLC grade) and enzyme at concentrations given in table and figure legends. Reaction mixtures were set up and incubated at 37 $^{\circ}\text{C}$ for time intervals given in table and figure legends. Reactions were stopped by heating the samples at 95 $^{\circ}\text{C}$ for 5 min and stored at -20°C . For HPLC analysis 20 μ l of sample were mixed with 200 μ l of TEAB (0.05 M) and analyzed. The analytical gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min, to 30% eluent B after 35 min and to 50% eluent B after 40 min. Reaction yields were calculated from HPLC peak areas considering differences in absorbance due to molar absorptivities ϵ increasing with increasing chain length. The ϵ_{260} values ($\text{M}^{-1}\text{cm}^{-1}$) of GpppA and ⁷MeGpppA were calculated as 26900 (11500_G + 15400_A) and 25500, respectively. ϵ_{260} values of G (11500), ⁷MeG (10100) and A (15400) were taken from <http://www.owcsarzy.net/emethod.htm> and (20). ϵ_{260} values of products were calculated using the nearest neighbor method (see <http://www.owcsarzy.net/emethod.htm> and (21)). ϵ_{260} of GpppACC was calculated as 31200 (11500_G + 21000_{ApC} + 14200_{CpC} – 7200_C). Values of methylated and longer products were calculated likewise. Yields of total product are given as percentage of substrate (cap analog) conversion. Additionally, from these values, yields were converted into pmol product, which can then be related to the amount of enzyme (pmol) used to generate the determined amount of product. Yields of single products are given in percentage of substrate (cap analog) conversion or in percentage of total product (explained in figure or table legends).

Preparative scale. Samples were set up in 200 μ l reaction volume containing T7 DNA primase reaction buffer, 10 mM DTT and 50 μ g/ml BSA, 5 mM CTP, 1 mM cap analog, 10 μ M DNA template and 4 μ M T7 DNA primase. Reactions were incubated at 37 $^{\circ}\text{C}$ during time intervals given in Table 3, stopped by heating at 95 $^{\circ}\text{C}$ for 5 min and stored at -20°C . Samples of 50 μ l were mixed with 200 μ l of TEAB (0.05 M) and analyzed. Preparative gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 30 min and to 30% after 45 min. After peak separation and collection, the samples were lyophilized three times and resuspended in water. Their purity was verified by HPLC, their concentration determined by measuring OD₂₆₀ values and their

molecular mass measured by mass spectrometry. Yields of preparative reactions are given as isolated yields based on measured amounts of products (absorbance measurements of pure products after lyophilization using molar absorptivities calculated as given above) and the amount of substrate used (Table 3). Samples were stored at -20°C .

Methyltransferase experiments

Dengue virus MTase domain. The reaction was set up in 50 μl reaction volume containing 40 mM Tris, pH 7.5, 5 mM DTT, 500 nM NS5MTase_{DV}, 2 μM GpppAC₃ and 20 μM AdoMet, incubated for 60 min at 30°C and stopped by immediate freezing. For HPLC analysis, the sample was mixed with 210 μl of TEAB (0.05 M) and analyzed. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min, and to 30% eluent B after 45 min. The product peak was purified and analyzed by mass spectrometry.

Human N7 MTase. Reactions were set up in 40 μl reaction volume containing 40 mM Tris, pH 7.5, 5 mM DTT, 500 nM hMTase, 2 μM capped RNA oligonucleotide and 10 μM AdoMet. Reactions were incubated for 30 min at 30°C and stopped by immediate freezing. For HPLC analysis, 40 μl of sample were mixed with 210 μl of TEAB (0.05 M) and analyzed. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min, and to 30% eluent B after 45 min. The product peak was purified and analyzed by mass spectrometry.

Analysis of GpppA₂OMeC₃ by enzymatic digestion and HPLC

A preparative reaction (3 \times 250 μl) was set up using the same conditions as described above for Dengue virus MTase except that 1 μM NS5MTase_{DV} was used and the reaction stopped after 8 h. Samples of 250 μl reaction mixture were mixed with 750 μl of TEAB (0.05 M) and injected into the HPLC system. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min and to 20% after 35 min. After peak separation and collection, the product was lyophilized three times. Product yield was around 1 nmol of pure, lyophilized GpppA₂OMeC₃. Then 0.5 nmol GpppA₂OMeC₃ was digested in 20 μl reaction volume containing 50 mM Tris, pH 8.5, 5 mM MgCl₂, 0.05 units of nucleotide pyrophosphatase type II from *Crotalus adamanteus* (Sigma, enzyme preparation contains side activity phosphodiesterase I, 0.005 units in 20 μl reaction volume) and 10 units of calf intestine phosphatase (New England Biolabs). Reaction was allowed to proceed for 11 min at 37°C and stopped by heating to 95°C for 5 min. The crude reaction mixture was then diluted with water (120 μl), filtered on Nanosep 3K omega (Pall Corporation) and centrifuged at 16 000 $\times g$ for 20 min. Washing of the filter was done by adding 60 μl of water followed by centrifugation at 16 000 $\times g$ for 10 min. The combined filtrates were

mixed with 200 μl of TEAB (0.05 M) and analyzed by HPLC without using the on-line cleaning procedure. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min and to 30% after 45 min.

RESULTS AND DISCUSSION

Synthesis of (⁷Me)GpppAC_n by T7 DNA primase

Bacteriophage T7 DNA primase-helicase is able to synthesize small RNA primers with the sequence pppA(C)_n ($n = 3, 4$) from DNA templates containing the recognition sequence ^{5'}GTC^{3'} in which the cytidine is cryptic (22). The initiation site being flexible in terms of the phosphate moiety of the initiating adenosine (23) can accommodate GpppA or ⁷MeGpppA allowing thus the production of capped RNA oligonucleotides (17). For this study, we used the N-terminal fragment of the protein (residues 1 to 271), which contains the N-terminal Zn-binding domain (residues 1 to 56) and the catalytic RNA polymerase domain (residues 71–245) connected by a flexible linker (18). The DNA primase fragment was efficiently expressed in *E. coli*, soluble and easily purified (see Material and Methods).

We started with a DNA template, CCCCGGGTCT₂₅ (transcribed sequence underlined) designed for the synthesis of AC₃. Instead of the initiating ATP, we used GpppA and ⁷MeGpppA in order to produce (⁷Me)GpppAC₃. Product formation was analyzed by reverse-phase chromatography on a HPLC system. An 'on-line cleaning' process (24,25) was used to remove unwanted proteic material from the sample. This technique allowed direct analysis of crude enzymatic reaction mixtures, with no significant loss of material. The sample was loaded onto the pre-column, thus eliminating proteic material while retaining the oligonucleotide mixture. After 5 min, the flow path was inverted and the substrates and produced capped RNAs were delivered onto the separating column by starting an acetonitrile gradient. Figure 1 shows the profiles after 6 h of primase reaction. Single peak material was purified and the molecular masses determined by mass spectrometry (see Material and Methods). Substrates GpppA and ⁷MeGpppA were efficiently incorporated by T7 DNA primase into small-capped RNAs (⁷Me)GpppAC_n ($1 \leq n \leq 4$). Apart from the main product (⁷Me)GpppAC₃, smaller products were generated, (⁷Me)GpppAC and to a lower extent (⁷Me)GpppAC₂ (not labeled in Figure 1), as well as a longer product, (⁷Me)GpppAC₄. The long DNA template size which was chosen albeit the primase fragment works equally on short templates (26), allowed its easy separation from the reaction products because of its longer retention time (38.3 min).

Subsequently, we followed product formation over time in order to reach optimal substrate conversion (Figure 2). Figure 2A shows the overall yield when GpppA or ⁷MeGpppA were used as cap analogs. T7 DNA primase incorporates both to same extent. Reactions were completed to 96% after 21 h. Figure 2B shows accumulation of individual products GpppAC to GpppAC₅ of one

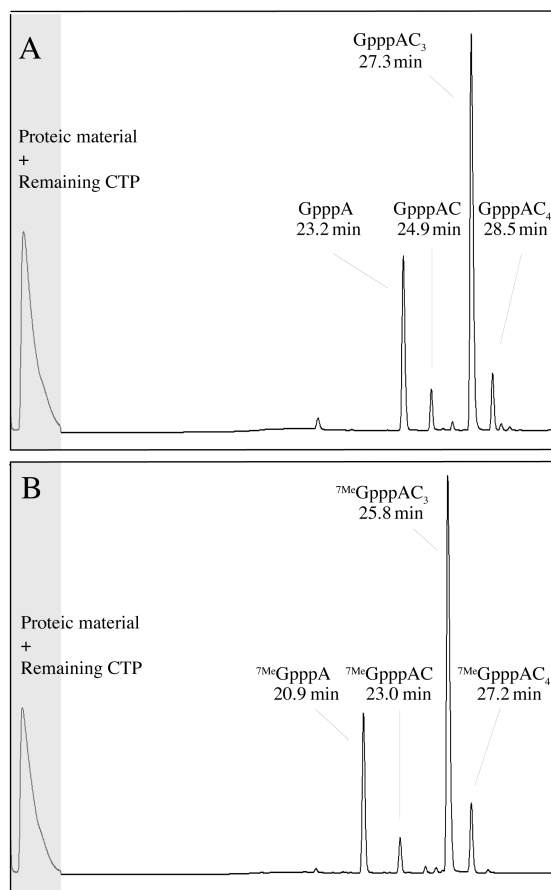


Figure 1. HPLC profiles of T7 DNA primase reaction mixtures using DNA template CCCC^{GGT}TCT₂₅. Crude enzymatic mixtures were analyzed directly. Reaction conditions were as follows: T7 DNA primase reaction buffer, DTT and BSA as given in Material and Methods, 5 mM CTP, 1 mM GpppA (A) or ⁷MeGpppA (B), 10 μM DNA template CCCC^{GGT}TCT₂₅ (named dAC3) and 4 μM T7 DNA primase. Reactions were incubated at 37°C for 6 h. The first section (marked in gray) denotes the removal of proteic material and remaining CTP by an on-line cleaning procedure using a pre-column. The produced capped RNAs were then separated on the reverse-phase C18 column. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min, reaching 30% after 35 min and 50% after 40 min.

of the reactions using GpppA as cap analog. GpppAC was produced during the early phase of reaction and completely converted into longer products after 16 h. GpppAC₂ was equally only present during the early phase of the reaction but to very low extent. After 1 h, GpppAC₃ was the main product and remained so throughout the reaction. GpppAC₄ appeared almost immediately and its yield grew linearly, whereas GpppAC₅ appeared after 15 h. Thus leaving the reaction longer opens the possibility to produce higher amounts of longer substrates (see below). The product profiles were similar when ⁷MeGpppA was used as cap analog (not shown).

Higher accumulation of AC than of AC₂ products has been observed repeatedly (27,28). Recently, it was proposed that the accumulation of the abortive AC product results from a change in conformation, namely

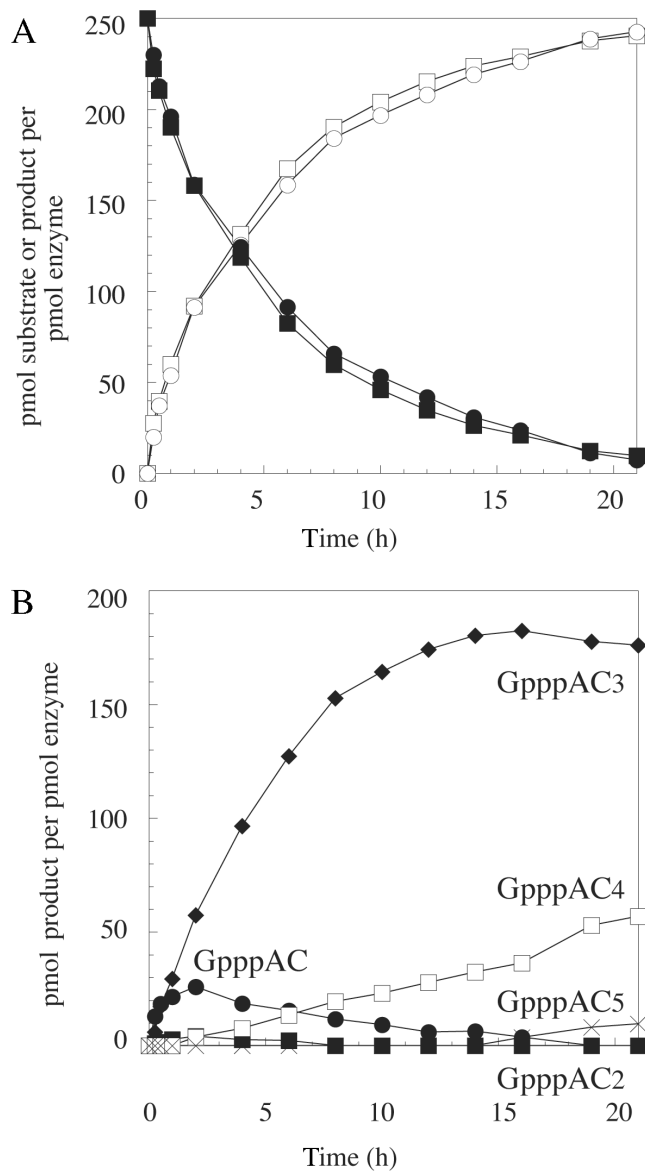


Figure 2. Time course of capped RNA synthesis by T7 DNA primase on DNA template dAC3 (CCCC^{GGT}TCT₂₅). Reaction conditions are given in the legend of Figure 1. Samples were taken at the given time points and analyzed by HPLC. The determination of yields (pmol product per pmol enzyme) is detailed in Material and Methods. (A) global analysis of substrate depletion (■ GpppA, ● ⁷MeGpppA) and product generation (□ GpppAC_n, ○ ⁷MeGpppAC_n) with time, (B) formation of individual products in the GpppA-capped RNA product mixture (● GpppAC, ■ GpppAC₂, ◆ GpppAC₃, □ GpppAC₄, × GpppAC₅).

in the relative position of the Zn-binding and RNA polymerase domains, occurring after the synthesis of the first phosphodiester bond so that the AC product is released more readily (29). Once the elongation-mode conformation is in place, longer products are formed. As it has been described before for non-capped RNA oligonucleotide synthesis by T7 DNA primase (22,27,28), the primase added extra non-templated cytidines and thus produced (⁷Me)GpppAC₄ and (⁷Me)GpppAC₅. This phenomenon might be due to either the ability of the primase to mis-incorporate CMP opposite

Table 1. Influence of DNA oligonucleotide dAC3 (CCCCGGGTCT₂₅), CTP and GpppA concentrations on T7 DNA primase reaction yield and product profile. Standard reaction conditions were used as given in the legend to Figure 1 corresponding to the presence of 250 pmol of cap analog substrate per pmol T7 DNA primase. The concentrations of DNA template and CTP/cap analog were then changed as given. Reactions were incubated for 21 h and analyzed by HPLC. Determination of yields of total product (pmol per pmol enzyme and percentage of substrate conversion) and of single products (percentage of total product) is explained in Material and Methods

Variable	Concentration (mM)	Total product GpppAC _n		GpppAC	GpppAC ₂	GpppAC ₃	GpppAC ₄	GpppAC ₅
		(pmol/pmol enzyme)	(%)					
DNA template	0.005	150.9	60.3	8.7	1.7	62.6	21.6	5.4
	0.01	203.0	81.2	3.4	0.6	61.9	27.4	6.7
	0.02	239.8	95.9	0.6	0.5	53.1	37.1	8.6
	0.05	249.0	99.6	0	4.2	38.9	47.4	9.4
CTP/GpppA	5/1	222.9	90.4	1.8	0	62.4	28.8	7.1
	10/2	130.5	26.1	30.3	4.8	54.9	10.0	0
	20/4	47.5	4.7	77.7	8.7	13.6	0	0
	10/1	65.3	26.2	31.3	5.1	52.2	10.0	1.4
	20/1	25.2	10.1	58.2	7.7	29.3	4.7	0

cytidine (27), the addition of a non-templated overhang (27) or pseudo-templated transcription where the primase reiteratively transcribes a small homopolymeric template stretch (30).

Variation of DNA template, CTP, cap analog and enzyme concentration

The initially used conditions for the production of capped RNA oligonucleotides were 5 mM CTP, 1 mM cap analog, 10 μM DNA template CCCCCGGGTCT₂₅ (named dAC3) and 4 μM T7 DNA primase. Routinely, we obtained overall conversions of 81–96% after 21 h. In view of the possibility to obtain a higher percentage of longer products maintaining overall yields approaching 100%, the influence of DNA template, CTP, cap analog and enzyme concentration was studied.

The DNA template concentration was varied between 5 and 50 μM. CTP and cap analog concentrations were not varied in total independence because a higher proportion of CTP than cap analog will always be incorporated into the products. The ratio GpppA to cytidine in product GpppAC₅ for instance is 5 to 1. We kept this ratio as a minimum and increased CTP and GpppA concentration together from the starting values 5 and 1 mM to 20 and 4 mM, respectively. Furthermore, we kept the cap analog concentration at 1 mM raising only the CTP concentration. Table 1 shows the results of optimization using cap analog GpppA, which were similar for ⁷MeGpppA reactions (not shown). Note that there is a difference in substrate conversion (81.2% versus 90.4%) between two identical reactions (0.01 mM DNA template and 5 mM CTP/1 mM GpppA, respectively), which reflect the variations we obtained between independent reaction series. Nevertheless product distribution was always very similar.

The increase in DNA template concentration up to 50 μM led to an increase in overall yield and product length. Thus 50 μM DNA template seems to be the concentration of choice. On the other hand, it has to be considered that template usage is much more efficient when lower concentrations are used. At 50 μM DNA

template concentration, *ca.* 20 pmol RNA oligonucleotide are produced from one pmol DNA template, i.e. the template is recycled 20 times supposing that all T7 DNA primase molecules are active. This can be calculated from the amount of total product generated (249.1 pmol per pmol primase) and the amount of DNA of template used in the reaction (12.5 pmol per pmol primase). When 5 and 10 μM DNA template is used, initiation happens 126 and 80 times, respectively, at each template molecule.

Increasing CTP and cap analog concentration at the same time maintaining a ratio of 5 to 1 resulted in a decrease in total yield and product length, thus both the initiation and elongation part of the primase reaction seem to be affected drastically. When we increased the CTP concentration alone, inhibition was stronger regarding product formation in absolute terms (pmol product per pmol enzyme) whereas product distribution essentially stayed the same. Inhibition by CTP may be explained by the formation of a dead-end complex by non-productive binding between the enzyme and CTP (31) that prevents productive binding of the cap analog. Activity seems to be partially rescued by higher concentrations of cap analog. However, higher cap analog concentrations result in lower overall substrate conversion. In conclusion, CTP and cap analog concentrations were kept at 5 mM and 1 mM, respectively.

Enzyme concentration was varied between 1 and 8 μM. As shown in Figure 3, the overall yield and the length of formed products increase with enzyme concentration within the tested range (upper panel). Nevertheless, the efficiency of the enzymatic reaction decreases from 867.5 pmol per pmol enzyme at 1 μM to 125.0 pmol per pmol enzyme at 8 μM enzyme concentration (lower panel). In conclusion, 4 μM T7 DNA primase was kept as enzyme concentration for oligonucleotide production on a preparative scale and further optimization studies.

Variation of DNA template length and reaction time

In order to produce a series of capped RNA oligonucleotides of different length, several DNA templates

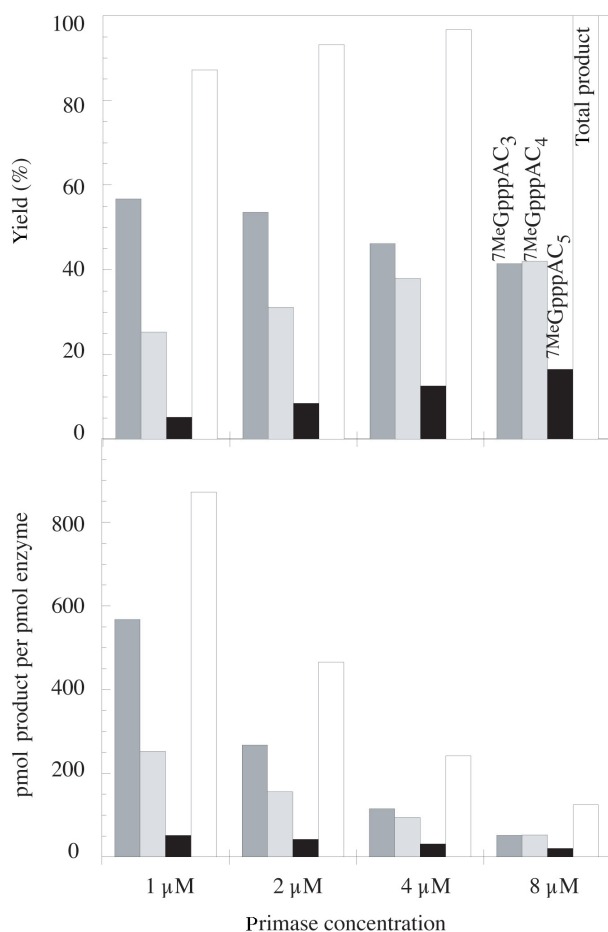


Figure 3. Influence of T7 DNA primase concentration on reaction yield and product profile. Standard reaction conditions were used as given in the legend of Figure 1 except for T7 DNA primase concentration which was varied between 1 and 8 μM corresponding to the presence of 1000 and 125 pmol of cap analog substrate ($^{7\text{Me}}\text{GpppA}$) per pmol T7 DNA primase. DNA template was dAC3 (CCCCGGTCT₂₅). Reactions were incubated for 21 h and analyzed by HPLC as described in Material and Methods. Yields in percentage conversion of cap analog into each single product (upper panel) and pmol product per pmol enzyme (lower panel) were determined as detailed in Material and Methods. Total product represents the sum of conversion into GpppAC₃, GpppAC₄ and GpppAC₅. Amounts of GpppAC and GpppAC₂ products were negligible under the tested reaction conditions.

Table 2. Influence of DNA template length and reaction time on T7 DNA primase reaction yield and product profile. Standard reaction conditions were those given in the legend of Figure 1 corresponding to the presence of 250 pmol of cap analog substrate per pmol T7 DNA primase. DNA templates dAC2 to dAC5 correspond to CCCCCGGTCT₂₅ to CCGGGGGTCT₂₅, respectively. Reactions were incubated at 37°C and analyzed by HPLC as described in Material and Methods. Products are listed with the sequence following the cap $^{7\text{Me}}\text{G}$, e.g. AC stands for $^{7\text{Me}}\text{GpppAC}$. Yields of total product (pmol per pmol enzyme and percentage conversion of substrate) and of single products (percentage of total product) were determined as explained in Material and Methods

DNA template	Reaction Time (h)	Total product $^{7\text{Me}}\text{GpppAC}_n$		AC									
		(pmol/pmol enzyme)	(%)	AC	AC ₂	AC ₃	AC ₄	AC ₅	AC ₆	AC ₇	AC ₈	AC ₉	
				(% of total product $^{7\text{Me}}\text{GpppAC}_n$)									
dAC2	21	250.0	100	0	75.2	21.2	3.6	0	0	0	0	0	0
dAC3	21	240.3	96.1	0	0	72.1	23.6	4.2	0	0	0	0	0
dAC4	21	195.3	78.1	4.6	0	31.8	44.7	15.8	3.1	0	0	0	0
	48	233.6	93.4	1.9	0.5	0	27.2	50.5	18.0	1.8	0	0	0
dAC5	21	139.2	55.7	17.8	6.5	4.9	0.4	21.1	30.4	15.7	3.1	0	0
	48	176.6	70.6	9.9	3.5	2.7	0.3	19.3	33.9	22.7	6.5	1.2	0
	71	179.3	71.7	9.1	3.1	1.6	0	13.5	35.3	27.3	8.2	1.9	0

($\text{C}_{(6-n)}\text{G}_n\text{GTCT}_{25}$, $1 \leq n \leq 4$) were used and the reaction time was varied. Table 2 shows that the use of templates dAC2 ($\text{C}_5\text{GGTCT}_{25}$) to dAC5 ($\text{C}_3\text{GGGGTCT}_{25}$) varying the reaction time between 21 and 71 h allowed the production of capped oligonucleotides $^{7\text{Me}}\text{GpppAC}$ to $^{7\text{Me}}\text{GpppAC}_9$. In accordance with Figure 2, almost identical results were obtained with GpppA as cap analog (not shown). Highest yields (100 and 96.1%, respectively) were obtained when DNA templates dAC2 and dAC3 were used. The main products after 21 h corresponded to $^{7\text{Me}}\text{GpppAC}_2$ (75.2%) and $^{7\text{Me}}\text{GpppAC}_3$ (72.1%), respectively. As observed before, considerable amount of $(n+1)$ products (21.2 and 23.6%, respectively) and small amounts of $(n+2)$ products (3.6% and 4.2%, respectively) were formed. With DNA templates dAC4 and dAC5, the overall yield of the reactions were lower (78.1 and 55.7%, respectively). Yields could be increased to a certain limit (93.4 and 70.6%, respectively) by extending the reaction time to 48 h. When dAC4 was used, the main product after 21 h was $^{7\text{Me}}\text{GpppAC}_4$ (44.7%) but the $(n+1)$ product $^{7\text{Me}}\text{GpppAC}_5$ after 48 h (50.5%). The $(n+1)$ product $^{7\text{Me}}\text{GpppAC}_6$ was the main product in the case of dAC5 after 21 h (30.4%) and remained so after 48 h (33.9%). Allowing the reaction using dAC5 to proceed further to 71 h led to a negligible increase in overall product yield and chain length. In conclusion, an adequate DNA template and reaction time of up to 48 h can be chosen to produce a desired spectrum of $(^{7\text{Me}})\text{GpppAC}_n$ ($1 \leq n \leq 9$). The chain length distribution of the capped RNA oligonucleotides is wider when DNA templates with longer guanine sequences are used (dAC4 and dAC5).

Addition of fresh T7 DNA primase and CTP

Using dAC5, it was tested if yield and product chain length could be increased by adding fresh enzyme after 48 h and leaving the reaction to 71 h. Additionally, CTP was added to replenish free CTP thus trying to allow synthesis of longer products but to avoid inhibition by high CTP concentrations (see above). The addition of enzyme alone increased the overall yield of the reaction and product chain length only to a limited extent (76.3% substrate conversion versus 73.7% without addition of

Table 3. Large-scale synthesis of short (^{7Me})GpppA-capped RNAs starting from 200 nmol of (^{7Me})GpppA. DNA templates dAC2, dAC3 and dAC4 correspond to CCCCCGGTCT₂₅, CCCC^{7Me}GGTCT₂₅ and CCC^{7Me}GGGTCT₂₅, respectively. For experimental conditions and MALDI-TOF characterization, see Materials and Methods. Yields are given as isolated yields (percentage of substrate conversion and nmol or μg product produced from 200 nmol of cap analog) based on absorbance measurements of pure products after lyophilization. Given retention times correspond to the use of the preparative gradient given in Material and Methods

Cap analog	DNA template/ Reaction time	HPLC retention time (min)	Purity (%)	Isolated yields			m/z exp. negative mode	m/z calc.
				(%)	(nmol)	(μg)		
GpppAC	dAC4/48 h	27.9	100	2	4	12.9	1076.22	1076.58
GpppAC ₂	dAC2/15 h	29.6	100	37	74	16.6	1382.23	1381.76
GpppAC ₃	dAC3/21 h	31.2	100	42	84	20.2	1688.24	1686.94
GpppAC ₄	dAC4/48 h	31.1	99	23	46	23.9	1991.42	1992.12
GpppAC ₅	dAC4/48 h	33.3	100	21	42	27.6	2297.55	2297.31
GpppAC ₆	dAC4/48 h	35.0	99	8	15	31.2	2601.81	2602.49
GpppAC ₇	dAC4/48 h	36.4	91	1	2	34.9	2906.83	2907.67
^{7Me} GpppAC	dAC4/48 h	26.7	99	2	3	13.1	1092.33	1090.61
^{7Me} GpppAC ₂	dAC2/15 h	28.7	100	31	61	16.8	1397.25	1395.79
^{7Me} GpppAC ₃	dAC3/21 h	30.7	99	52	103	20.4	1701.58	1700.97
^{7Me} GpppAC ₄	dAC4/48 h	31.9	100	13	26	24.1	2006.91	2006.15
^{7Me} GpppAC ₅	dAC4/48 h	32.9	100	34	68	27.7	2311.68	2311.33
^{7Me} GpppAC ₆	dAC4/48 h	34.5	100	17	33	31.4	2616.77	2616.51
^{7Me} GpppAC ₇	dAC4/48 h	36.0	98	2	3	35.1	2921.68	2921.69

enzyme). When enzyme and CTP were added at the same time (the resulting CTP concentration was adjusted to 2 mM supposing that CTP had been consumed completely), the overall yield decreased to 68.0% substrate conversion but the product chain length was shifted to longer products. After 71 h, ^{7Me}GpppAC₈ represented 18% of total product instead of 8.2% without addition of enzyme and CTP. When the amount of added CTP was further increased (adjusted to 5 mM concentration), the reaction yield decreased further without increasing the product length in comparison to 2 mM. Therefore if longer products are desired, the addition of enzyme and CTP up to 2 mM is beneficial.

Synthesis of (^{7Me})GpppAC_n on a preparative scale

Large-scale reactions (200 μl reaction volume) were set up using the optimized conditions described above, but trying to use reasonable concentrations of enzyme and HPLC grade DNA template (4 and 10 μM, respectively). Globally, no significant differences in activity and product distribution were observed between small (20 μl) and large (200 μl) scale reactions (not shown). Parallel reactions were set up to further increase production scale. We used the DNA templates dAC2 and dAC3 for the production of (^{7Me})GpppAC₂ and (^{7Me})GpppAC₃, respectively, whereas dAC4 was used as the template for the production of (^{7Me})GpppAC₄ to (^{7Me})GpppAC₇. (^{7Me})GpppAC was purified as a minor product of the dAC4 reaction. The reaction times were chosen to ensure the best conversion rate of the starting material while avoiding excessive distribution of the products, especially for the shorter capped RNAs. After purification, products were lyophilized three times to remove the volatile buffer solution. Each compound was characterized by mass spectrometry and its purity confirmed by HPLC (Table 3).

The isolated yields of each product after lyophilization are given in Table 3. The best yields (42 and 52%) were

obtained for the dAC3 reactions where also a good balance between conversion rate of the starting material and low dispersion of the products could be obtained. Slightly lower yields were obtained for the dAC2 reactions. As expected the global dispersion of the products for the dAC4 series was the widest with the advantage that this reaction afforded reasonable amounts of (^{7Me})GpppAC and (^{7Me})GpppAC₄ to (^{7Me})GpppAC₇ to be produced. Thus we were able to produce a range of short GpppA- or ^{7Me}GpppA-capped RNA molecules with yields up to 52% corresponding to ca. 100 nmol (corresponding to ca. 20 μg) of product ^{7Me}GpppAC₃ starting from 200 nmol cap analog. The obtained yields may still be increased when higher DNA template and enzyme concentrations are used and, concerning longer products, when enzyme and CTP are added after 48 h (see above).

Chemical approaches (the advantage being that the sequence can be varied) for the synthesis of short-capped RNAs described in the literature gave isolated yields from 2 to 37%, as summarized by Koukhareva (8). Similar or superior results have been obtained, at least for the main products, using our enzymatic approach. Other strategies to produce GpppA-capped RNA oligonucleotides, which combine chemical synthesis of diphosphorylated RNA followed by enzymatic addition of the cap by commercially available guanylyltransferase (9), or use two enzymatic reactions (synthesis of triphosphate poly(rA) (32) followed by capping with guanylyltransferase (10)), produced radiolabeled products for which no product yields were given. Concerning one-pot enzymatic reactions, *E. coli* as well as bacteriophage T7, SP6 and T3 DdRps using their promoter sequences were reported to have produced GpppA-capped RNA in microgram range (12,13,16). Nevertheless 1 μg of one these long transcripts of ca. 2200 nucleotides (12) correspond to 65 fmol of capped RNA and thus they are useful for

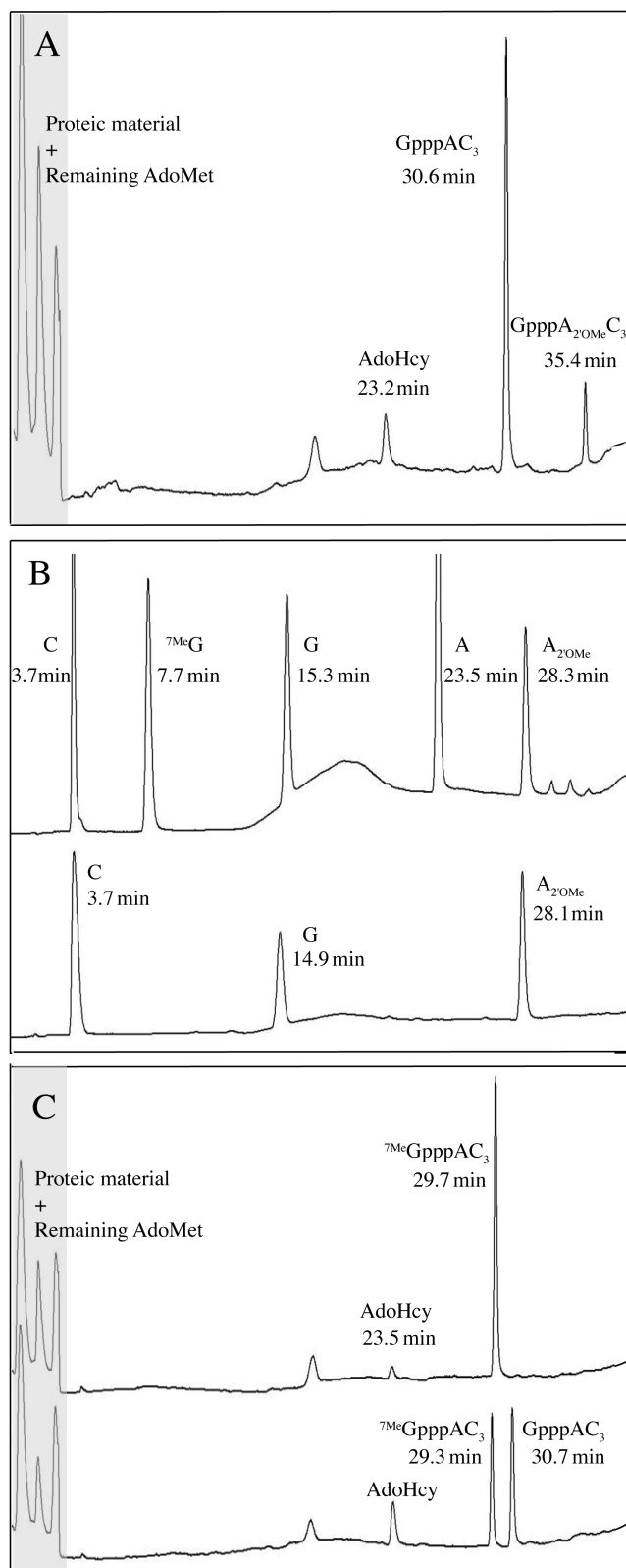


Figure 4. Methyltransferase reactions using GpppAC₃ as substrate. (A): HPLC profile of the NS5MTase_{DV} reaction mixture using GpppAC₃ as substrate. Reaction conditions are given in Material and Methods. The crude enzymatic mixture was analyzed without sample treatment. The first section (in gray) indicates the removal of proteic material and remaining AdoMet by on-line cleaning on the pre-column

cellular transfection assays but not as purified substrates in enzymatic assays. Very recently, the T7 class II $\phi 2.5$ promoter (14) was successfully used with T7 DdRp to produce semi-purified GpppA-capped RNA transcripts of *ca.* 180 nucleotides. No product yields were given. The capped RNAs served as substrates in multiple-step, radioactive *Flavivirus* MTase assays (15). This approach offers the advantage that sequence-specific RNA molecules can be generated. It will be interesting to see if it allows production of sufficient amounts for wider biochemical, inhibition and crystallographic studies.

GpppA- and ⁷MeGpppA-capped RNAs as methyltransferase substrates

The purified capped RNA oligonucleotides were tested as substrates of Dengue 2'OMTase, expressed as recombinant N-terminal domain of protein NS5 (NS5MTase_{DV}) in *E. coli* (6). In contrast to the usual set-up of methyltransfer assays (15), we used non-radioactive AdoMet and RNA substrate. The reaction mixture was analyzed by reverse-phase HPLC including an on-line cleaning procedure without any additional sample treatment. Figure 4A shows the resulting profile after a 30-min reaction using GpppAC₃ as substrate. The retention time of substrate GpppAC₃ was 30.6 min. An additional peak was observed at 35.4 min. Its molecular mass was determined by mass spectrometry as being 1702.44 (*m/z*, [M-H]⁻) compared to 1688.24 (*m/z*, [M-H]⁻, see Table 3) of GpppAC₃, thus one methyl group was transferred. In order to identify the receiving position, we digested the product at 35.4 min by a mix of nucleotide pyrophosphatase, phosphodiesterase I and calf intestine phosphatase rendering the corresponding nucleosides. The resulting mixture was analyzed by HPLC (Figure 4B, lower chromatogram). The comparison with standard compounds (upper chromatogram), which was verified by co-injection (not shown), allowed the identification of 2'O position of the adenosine as the methylated position. Thus, in accordance with earlier results using non-purified substrates (6), we found that the product of methyltransfer by NS5MTase_{DV} using substrate GpppAC₃ corresponds to GpppA_{2'OMe}C₃. Note that the important delay in elution of GpppA_{2'OMe}C₃ in comparison to substrate GpppAC₃ is caused by an increase in hydrophobicity due to the methylation of the 2'OH group combined with the use of a very shallow

(see Material and Methods). The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min reaching 30% after 45 min. (B): HPLC profile (lower chromatogram) of nucleoside product mixture after enzymatic digestion of the product generated by methylation of GpppAC₃ by NS5MTase_{DV} (see panel A at 35.4 min). Enzymatic digestion was done using a mix of nucleotide pyrophosphatase, phosphodiesterase I and calf intestine phosphatase. The upper chromatogram shows a mixture of standard compounds. No on-line cleaning was used. The gradient started after 5 min at 100% eluent A with an increase to 10% eluent B after 25 min and to 30% after 45 min. (C): HPLC profile of the human mRNA cap N7MTase reaction mixture using GpppAC₃ as substrate (lower chromatogram) in comparison to ⁷MeGpppAC₃ being used as a control substrate (upper chromatogram). Reaction conditions are given in Material and Methods. The crude enzymatic mixtures were analyzed without sample treatment as described in the legend of panel A.

gradient (see figure legend). In order to see if methylation could also be achieved at the guanine-N7 position of GpppAC₃, we used recombinant human mRNA cap N7MTase (33,34). The HPLC analysis of the reaction products (Figure 4C, lower chromatogram) in comparison to a control reaction using ⁷MeGpppAC₃ (upper chromatogram) shows that GpppAC₃ is indeed efficiently methylated at the guanine-N7 position. Note that in this case, a positive charge is generated upon methylation leading to a shorter elution time of the product (29.3 min) in comparison to the non-methylated substrate (30.7 min). Thus both methyltransfer reactions were readily detectable by our HPLC separation method. They can be easily analyzed in a quantitative way in terms of picomoles methyl group transferred by one picomole enzyme.

CONCLUSION

We were able to produce and purify a series of GpppA-capped RNA oligonucleotides of different length, (⁷Me)GpppAC_n (1 ≤ n ≤ 9), in a simple one-step enzymatic reaction using a recombinant bacteriophage T7 DNA primase fragment. Our yields (Table 2) correspond well to the yield of 75% substrate conversion obtained upon production of ⁷MeGpppACC using the full-length T7 DNA primase-helicase (17). In our study, we went further in optimizing the production of longer molecules using just the T7 DNA primase fragment. The isolated yields of products (⁷Me)GpppAC_n (1 ≤ n ≤ 7) obtained after large-scale synthesis and purification, in average 99% (Table 3), are superior or comparable to other methods, the additional advantage of the method presented here being the simplicity at least for biochemical laboratories with protein expression facilities. Moreover, this method allows the production of highly purified and easily quantified capped RNAs of different lengths for (1) non-radioactive activity assays, (2) studies of methyltransferase specificity in relation to substrate length and (3) co-crystallization studies of a variety of eukaryotic and viral methyltransferases and guanylyltransferases.

We showed that our purified capped RNA oligonucleotides serve as substrate of a mRNA cap N7MTase and a 2'OMTases of human origin and Dengue virus, respectively. Both methyltransfer reactions were monitored by a non-radioactive, quantitative HPLC assay.

In addition, our optimized method allows also the production of di- or triphosphate RNA oligonucleotides (using ATP or ADP instead of GpppA), which are valuable tools for the biochemical and structural studies of RNA triphosphatases and guanylyltransferases. Besides, preliminary tests have shown that the production of GpppACUC_n and possibly other RNA oligonucleotides varying at positions 3 and further are attainable (not shown).

ACKNOWLEDGEMENTS

We wish to thank Charles C. Richardson (Harvard Medical School, Boston, USA) and Aaron J. Shatkin (Center of Advanced Biotechnology and Medicine, Piscataway, USA)

for kindly providing the T7 DNA primase expression vector and the cDNA of human mRNA cap N7MTase, respectively. We thank David Karlin for the production of the T7 DNA primase as well as Marie-Pierre Egloff for purified NSSMTase_{DV}. This study was supported, in part, by the integrated project VIZIER (LSHG-CT-2004-511960) of the European Union 6th PCRDT. F.P. was supported by a postdoctoral fellowship from the Centre National de la Recherche Scientifique (CNRS), France. Funding to pay the Open Access publication charge was provided by CNRS.

REFERENCES

1. Furuichi, Y. and Shatkin, A.J. (2000) Viral and cellular mRNA capping: past and prospects. *Adv. Virus Res.*, **55**, 135–184.
2. Shuman, S. (2001) Structure, mechanism, and evolution of the mRNA capping apparatus. *Prog. Nucleic Acid Res. Mol. Biol.*, **66**, 1–40.
3. Gould, E.A., de Lamballerie, X., Zanotto, P.M. and Holmes, E.C. (2003) Origins, evolution, and vector/host coadaptations within the genus Flavivirus. *Adv. Virus Res.*, **59**, 277–314.
4. Mackenzie, J.S., Gubler, D.J. and Petersen, L.R. (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.*, **10**, S98–109.
5. Benarroch, D., Egloff, M.-P., Mulard, L., Guerreiro, C., Romette, J.-L. and Canard, B. (2004) A structural basis for the inhibition of the NS5 Dengue virus mRNA 2'-O-methyltransferase domain by ribavirin 5'-triphosphate. *J. Biol. Chem.*, **279**, 35368–35643.
6. Egloff, M.-P., Benarroch, D., Selisko, B., Romette, J.-L. and Canard, B. (2002) An RNA cap (nucleoside-2'-O)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. *EMBO J.*, **21**, 2757–2768.
7. Jemielity, J., Heinonen, P., Lonnberg, H. and Darzynkiewicz, E. (2005) A novel approach to solid phase chemical synthesis of oligonucleotide mRNA cap analogs. *Nucleosides, Nucleotides & Nucleic Acids*, **24**, 601–605.
8. Koukhareva, I.I. and Lebedev, A.V. (2004) Chemical route to the capped RNAs. *Nucleosides, Nucleotides & Nucleic Acids*, **23**, 1667–1680.
9. Brownlee, G.G., Fodor, E., Pritlove, D.C., Gould, K.G. and Dalluge, J.J. (1995) Solid phase synthesis of 5'-diphosphorylated oligoribonucleotides and their conversion to capped m7Gppp-oligoribonucleotides for use as primers for influenza A virus RNA polymerase in vitro. *Nucleic Acids Res.*, **23**, 2641–2647.
10. Shuman, S. (1989) Functional domains of vaccinia virus mRNA capping enzyme. Analysis by limited tryptic digestion. *J. Biol. Chem.*, **264**, 9690–9695.
11. Lockless, S.W., Cheng, H.-T., Hodel, A.E., Quijcho, F.A. and Gershon, P.D. (1998) Recognition of capped RNA substrates by VP39, the vaccinia virus-encoded mRNA cap-specific 2'-O-methyltransferase. *Biochemistry*, **37**, 8564–8574.
12. Holden, K.L. and Harris, E. (2004) Enhancement of dengue virus translation: role of the 3' untranslated region and the terminal 3' stem-loop domain. *Virology*, **329**, 119–133.
13. Yun, S.I., Kim, S.Y., Rice, C.M. and Lee, Y.M. (2003) Development and application of a reverse genetics system for Japanese encephalitis virus. *J. Virol.*, **77**, 6450–6465.
14. Coleman, T.M., Wang, G. and Huang, F. (2004) Superior 5' homogeneity of RNA from ATP-initiated transcription under the T7 phi 2.5 promoter. *Nucleic Acids Res.*, **32**, e14.
15. Ray, D., Shah, A., Tilgner, M., Guo, Y., Zhao, Y., Dong, H., Deas, T.S., Zhou, Y., Li, H. and Shi, P.Y. (2006) West Nile virus 5'-cap structure is formed by sequential Guanine N-7 and ribose 2'-O methylations by nonstructural protein 5. *J. Virol.*, **80**, 8362–8370.
16. Contreras, R., Cheroutre, H., Degrave, W. and Fiers, W. (1982) Simple, efficient in vitro synthesis of capped RNA useful for direct expression of cloned eukaryotic genes. *Nucleic Acids Res.*, **10**, 6353–6362.
17. Matsuo, H., Moriguchi, T., Takagi, T., Kusakabe, T., Buratowski, S., Sekine, M., Kyogoku, Y. and Wagner, G. (2000) Efficient synthesis of

- ¹³C,¹⁵N-labeled RNA containing the cap structure m⁷GpppA. *J. Am. Chem. Soc.*, **122**, 2417–2421.
18. Kato, M., Ito, T., Wagner, G., Richardson, C.C. and Ellenberger, T. (2003) Modular architecture of the bacteriophage T7 primase couples RNA primer synthesis to DNA synthesis. *Mol. Cell*, **11**, 1349–1360.
 19. Frick, D.N., Baradaran, K. and Richardson, C.C. (1998) An N-terminal fragment of the gene 4 helicase/primase of bacteriophage T7 retains primase activity in the absence of helicase activity. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 7957–7962.
 20. Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) *Data for Biochemical Research*, Oxford University Press, Oxford.
 21. Cantor, C.R., Warshaw, M.M. and Shapiro, H. (1970) Oligonucleotide interactions. 3. Circular dichroism studies of the conformation of deoxyoligonucleotides. *Biopolymers*, **9**, 1059–1077.
 22. Mendelman, L.V. and Richardson, C.C. (1991) Requirements for primer synthesis by bacteriophages T7 63-kDa gene 4 protein. *J. Biol. Chem.*, **266**, 23240–23250.
 23. Kusakabe, T. and Richardson, C.C. (1997) Gene 4 DNA primase of bacteriophage T7 mediates the annealing and extension of ribo-oligonucleotides at primase recognition sites. *J. Biol. Chem.*, **272**, 12446–12453.
 24. Pompon, A., Lefebvre, I., Imbach, J.-L., Kahn, S. and Farquhar, D. (1994) Decomposition pathways of the mono- and bis(pivaloyloxy methyl) esters of azidothymidine 5'-monophosphate in cell extract and in tissue culture medium: an application of the 'on-line IRSP-cleaning' HPLC technique. *Antivir. Chem. Chemother.*, **5**, 91–98.
 25. Pompon, A., Lefebvre, I. and Imbach, J.L. (1992) 'On-line internal surface reversed-phase cleaning': the direct HPLC analysis of crude biological samples. Application to the kinetics of degradation of oligonucleotides in cell culture medium. *Biochem. Pharmacol.*, **43**, 1769–1775.
 26. Frick, D.N. and Richardson, C.C. (1999) Interaction of bacteriophage T7 gene 4 primase with its template recognition site. *J. Biol. Chem.*, **274**, 35889–35898.
 27. Kusakabe, T. and Richardson, C.C. (1997) Template recognition and ribonucleotide specificity of the DNA primase of bacteriophage T7. *J. Biol. Chem.*, **272**, 5943–5951.
 28. Frick, D.N., Kumar, S. and Richardson, C.C. (1999) Interaction of ribonucleotide triphosphates with the gene 4 primase of bacteriophage T7. *J. Biol. Chem.*, **274**, 35899–35907.
 29. Qimron, U., Lee, S.J., Hamdan, S.M. and Richardson, C.C. (2006) Primer initiation and extension by T7 DNA primase. *EMBO J.*, **25**, 2199–2208.
 30. Jacques, J.P. and Kolakofsky, D. (1991) Pseudo-templated transcription in prokaryotic and eukaryotic organisms. *Genes Dev.*, **5**, 707–713.
 31. Cornish-Bowden, A. (2004) *Fundamentals of Enzyme Kinetics*. 3rd Edn., Portland Press, Oxford.
 32. Venkatesan, S. and Moss, B. (1980) Donor and acceptor specificities of HeLa cell mRNA guanylyltransferase. *J. Biol. Chem.*, **255**, 2835–2842.
 33. Pillutla, R.C., Yue, Z., Maldonado, E. and Shatkin, A.J. (1998) Recombinant human mRNA cap methyltransferase binds capping enzyme/RNA polymerase II complexes. *J. Biol. Chem.*, **273**, 21443–21446.
 34. Saha, N., Schwer, B. and Shuman, S. (1999) Characterization of human, *Schizosaccharomyces pombe*, and *Candida albicans* mRNA cap methyltransferases and complete replacement of the yeast capping apparatus by mammalian enzymes. *J. Biol. Chem.*, **274**, 16553–16562.