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Solid phase synthesis of 5′-diphosphorylated oligoribonucleotides and their conversion to capped m^{7}Gppp-oligoribonucleotides for use as primers for influenza A virus RNA polymerase *in vitro*

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ABSTRACT

We have synthesized four different 5′-diphosphorylated oligoribonucleotides, varying in length from 11 to 13 nucleotides by a new solid phase method. After deprotection and partial purification the 5′-diphosphorylated oligoribonucleotides could be converted to capped (m^{7}Gppp)-oligoribonucleotides using guanylyl transferase. Radiolabelled capped oligoribonucleotides acted as primers for the influenza A virus RNA polymerase *in vitro*. The solid phase method described here should also allow the addition of 5′-diphosphates to synthetic oligodeoxyribonucleotides and be capable of automation.

INTRODUCTION

The control of transcription and replication of the RNA genome of influenza A viruses is of great interest, not only because of its fundamental importance for the viral life cycle, but also because a fuller understanding of the mechanisms offer, in theory, the possibility of devising specific antiviral agents. A peculiarity of the influenza transcriptional mechanism is that mRNA synthesis is initiated at each of the eight negative-stranded RNA segments by the endonucleolytic cleavage of host precursor mRNA in the nucleus of infected cells by a specific endonuclease activity of the influenza-specific RNA polymerase (for reviews see 1,2). This recognizes capped ends of mRNA and cleaves predominantly, but not exclusively, at purine residues 9–15 nucleotides (nt) from the cap structure. The resultant capped oligonucleotide then acts as a primer by initiating influenza mRNA synthesis at the second or third nucleotide of the template virion RNA (3). A recent study has confirmed earlier data that priming by capped oligonucleotides can be uncoupled from the endonuclease activity of the influenza RNA polymerase (4).

The purpose of the present paper is to present an improved method of preparing capped oligonucleotides for the study of influenza virus transcription. Instead of synthesizing RNA *in vitro* by transcription of a suitable plasmid DNA to give 5′-triphosphorylated RNA, which is then capped by guanylyl transferase in the presence of GTP and *S*-adenosylmethionine (4), we use protected chemically synthesized oligoribonucleotides attached to a solid phase support as starting material for a chemical phosphorylation. After deprotection, capping is carried out enzymatically with guanylyl transferase (see Fig. 1 for overall scheme). A previous report from this laboratory (5) has described the use of chemically synthesized ppGmAAUACUCAAG, its capping and subsequent use as a primer for influenza RNA polymerase. However, no details of the phosphorylation method or characterization of the diphosphorylated oligonucleotide were presented.

The phosphorylation method used here is adapted from that of Ludwig and Eckstein (6) for synthesis of thioATP analogues from adenosine using the phosphitylating agent 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite) and subsequent reaction with pyrophosphate followed by oxidation with iodine. It had previously been adapted to the solid phase synthesis of 2′-*O*-methylated ATP from 2′-*O*-methylated adenosine attached to controlled pore glass (CPG) (7).

MATERIALS AND METHODS

Synthesis and deprotection of oligoribonucleotides

5′-Dimethoxytrityl 2′-*O*-t-butyldimethylsilyl cyanoethyl phosphoramidite-protected nucleosides and CPG 2′-*O*-t-butyldimethylsilyl-protected nucleosides were purchased from Peninsula Laboratories. These nucleosides were base-protected as follows: A and C, benzoyl; G, isobutyryl. 2′-*O*-Methyl cyanoethyl phosphoramidite dimethylformamidine-protected G was purchased from Glen Research. Oligoribonucleotide synthesis (0.2 μmol) was performed on an ABI 394 synthesizer using a standard RNA synthesis cycle with a 10 min coupling time with tetrazole. The following oligoribonucleotides were synthesized: GAAUACUCAAG, GmAAUACUCAAG (where Gm is 2′-*O*-methylguanosine), ACACUUGCUUUUG and U_{11}. Automatic deprotection of the 5′-dimethoxytrityl group was carried out on the ABI machine. All other protecting groups were removed manually using procedures slightly modified from Lamond and Sproat (8). Either anhydrous ethanolamine:anhydrous ethanol (1:1) at 60°C for 3 h or 0.88 sp. gravity ammonia:ethanol (1:3) at 60°C for 16 h was

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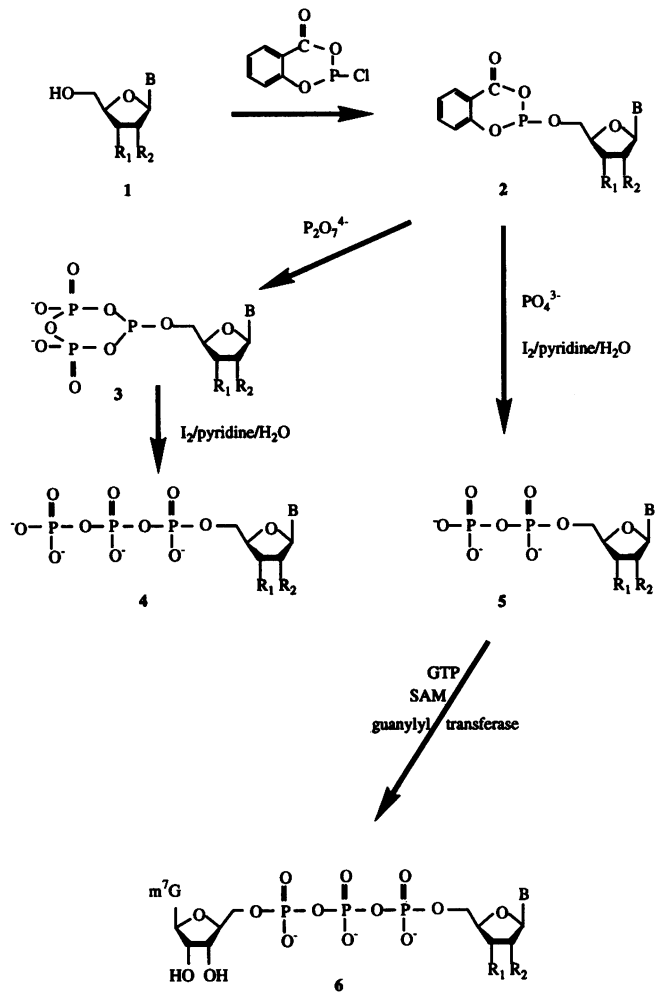

Figure 1. General scheme of the phosphorylation and capping method. This shows the predicted synthesis of triphosphates (6) and the proposed mechanism of diphosphate addition. R_1, cyanoethyl phosphotriester-linked oligoribonucleotide (base- and 2′-protected) attached to controlled pore glass; R_2, t-butyldimethylsilyl or methyl; B, protected base (see Materials and Methods).

used for base deprotection, hydrolysis of cyanoethyl groups and the succinyl linkage of the oligonucleotide to the CPG. After freeze drying to remove ethanol and ammonia, the 2′-*O*-t-butyldimethylsilyl protecting group was removed with 0.25 ml 1 M tetrabutylammonium fluoride in THF (Aldrich; kept over a molecular sieve, type 3A, to reduce the water content; 9) for 24 h at 30°C. After adding an equal volume of water, the fully deprotected oligonucleotide was desalted on a 3.5 ml Sephadex G-25 column (Pharmacia NAP-10, prepacked) in deionized water. The major $A_{260\,nm}$ fractions of 0.25 ml were pooled, freeze dried and redissolved in 50 µl 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.

Phosphorylation of oligoribonucleotides

Phosphorylation was carried out at room temperature on fully protected oligoribonucleotides attached to CPG after automated removal of the 5′-dimethoxytrityl group on the ABI synthesizer. The glass beads were then transferred to a small glass column (20 × 6 mm internal diameter) containing a sinter to trap the beads (Omnifit, Cambridge). The column could be maintained under near anhydrous conditions by an inlet septum through which reagents and dry solvents were injected via a 0.5 ml Hamilton syringe. Another inlet tube was connected via a drying tube containing silica gel to a N_2 cylinder via a valve, which was opened manually when reagents or solvents were to be removed from the column under N_2 pressure, to waste via another valve. The following protocol is slightly modified from Gaur *et al.* (7; Fig. 1), principally because of the much smaller scale of synthesis used (≤1 µmol oligonucleotide here, compared with 100 µmol nucleoside in Gaur *et al.*). The oligonucleotide-derivatized CPG (0.2–1 µmol) in the reaction column was initially washed three times with dry acetonitrile and then 0.3 ml dry pyridine:dioxan (1:3) was added, followed immediately by 0.1 ml 1 M 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite; Aldrich), freshly made up in dry dioxan. The column was inverted three times to mix the solution with the solid support and left for 15 min to form the bifunctional reactive intermediate **2**. The phosphitylating agent was then removed and the column washed three times with dry dioxan followed by three times with dry acetonitrile. Then 0.3 ml 0.5 M tri-*n*-butylammonium pyrophosphate (6) in dry dimethyl formamide (DMF) was added, followed immediately by 0.1 ml tri-*n*-butylamine. The column was again inverted three times to mix the reaction components. After 20 min the excess pyrophosphate was removed and the support washed three times with dry DMF, followed by three times with acetonitrile. Oxidation was then performed with iodine:H_2O:pyridine:THF (3:2:20:75) for 10 min, followed by washing three times with acetonitrile to remove excess iodine. Finally, the oligonucleotide was dried by passing through a stream of N_2 for 10 min. After removal of the derivatized support from the reaction vessel, deprotection was carried out using ammonia:ethanol for base deprotection, followed by desilylation and desalting on NAP-10 columns and freeze drying, as described (see above) for deprotection of oligoribonucleotides. This preparation is referred to as the crude phosphorylated oligonucleotide.

^{31}P NMR

Prior to ^{31}P NMR, DMF was removed from the tri-*n*-butylammonium pyrophosphate by evaporation *in vacuo* and the oil redissolved in water. Residual DMF was removed by re-evaporation from water twice more. The aqueous pyrophosphate solution (~0.2 M) was titrated to pH 10.0 with NaOH and compared with external standards of disodium hydrogen phosphate and tetrasodium pyrophosphate, also at pH 10.0, to avoid the significant variation in chemical shifts seen on ^{31}P NMR caused by the different phosphate ionization states at different pH values. ^{31}P NMR was performed on a Bruker AM250 instrument operating at 101.3 MHz, equipped with a 10 mm broad-band probe. Samples were placed in 8 mm diameter tubes which were then housed in 10 mm tubes for analysis, with the region between the two containing D_2O for field-frequency locking. Data were acquired at ambient probe temperatures (22°C) and spectra were referenced externally to 80% H_3PO_4 at 0.0 p.p.m. At least three compounds were present: pyrophosphate (–4.8 p.p.m., 51% yield), phosphate (3.7 p.p.m., 22% yield), an unassigned doublet closely associated with pyrophosphate (–4.1 p.p.m., 19% yield) and another unassigned multiplet (–18.5 p.p.m., 8% yield).

Electrospray ionization mass spectrometry

Crude phosphorylated deprotected oligoribonucleotides (~50 nmol derived from GmAAUACUCAAG or U_{11}) were electrophoresed

on a 20% polyacrylamide gel in 7 M urea in 1× Tris–borate–EDTA (TBE) buffer and the main optical product in each case (detected by UV shadowing) was cut out, crushed and eluted with 1.0 ml 0.25 M ammonium acetate for 16 h at 4°C with gentle shaking. After desalting on NAP-10 columns (see above) in water the oligonucleotides were dried *in vacuo* and redissolved in 0.2 ml water and desalted again to remove traces of cations which interfere in the mass spectrometry (10). The samples (~0.5 nmol) were then dissolved in 50 µl water. An aliquot of each of these samples was diluted to a concentration of 10 pmol/µl in a solution of 50% aqueous methanol containing 1% triethylamine for analysis by mass spectrometry as described (11). Briefly, electrospray ionization spectra of these samples were acquired using a PE Sciex (Norwalk, CT) API III+ triple quadrupole mass spectrometer. Mass analysis was made using only Q1 [calibrated in negative ion mode with d(CCCCCC)], analysing over the mass range 450–1600.

Capping and ^{32}P-labelling of phosphorylated oligonucleotides

Capping of the four crude phosphorylated oligonucleotides (~0.2 nmol) to give m^7G^{32}ppp-labelled oligonucleotides was achieved using 1 U guanylyl transferase (Gibco BRL) and 1 µM [α-^{32}P]GTP (3000 Ci/mmol; Amersham) in 0.05 M Tris–HCl, pH 7.8, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM dithiothreitol, 20 U human placental ribonuclease inhibitor (Promega), 0.1 mM *S*-adenosylmethionine in a 5 µl reaction volume for 1 h at 37°C. In some experiments bovine serum albumin (0.4 µg) was added. The reaction products were analysed, or in preparative experiments purified, by electrophoresis on 20% polyacrylamide–7 M urea gels. The major radioactive band was detected by autoradiography and eluted in 0.25 M ammonium acetate, as above. The eluate was centrifuged to remove gel pieces and the RNA precipitated from the supernatant with 3 vol ethanol in the presence of 2 M ammonium acetate and 20 µg yeast carrier RNA.

Other analytical methods

P1 nuclease (Boehringer) digestion was carried out in 30 mM ammonium acetate, pH 5.3, 10 mM $ZnSO_4$ (12) for 30 min at 37°C using 30 µg yeast RNA to establish the enzyme concentration needed for complete hydrolysis to 5′-mononucleotides, as judged by TLC on a Macherey-Nagel polygram SIL G/UV254 sheet (Camlab) developed using propan-2-ol:H_2O:ammonia (70:30:1) with detection of nucleotides under a UV lamp. Phosphorylated gel-purified ACACUUGCUUUUG (2.5 µg) and non-phosphorylated ACACUUGCUUUUG were then digested with P1 nuclease in a 10 µl reaction volume and, after checking by TLC on an aliquot that the digestion was complete, were analysed by HPLC (Beckman Gold) using a Beckman Spherogel-TSK DEAE-5PW (10 µm particle size, 7.5 × 75 mm) anion-exchange column using a gradient from 10 mM to 0.5 M triethylamine acetate, pH 6.8. P1 nuclease cleaves oligonucleotides and 2′-*O*-methylated residues to give mononucleoside 5′-phosphate end products. A 5′-triphosphorylated end group would degrade to give pppN.

T_2 RNase (Sigma) digestion of crude phosphorylated (30 µg) and control non-phosphorylated ACACUUGCUUUUG (30 µg) was carried out in 0.05 M ammonium acetate, pH 4.5, with 2 U/ml enzyme for 2 h at 37°C, followed by analysis by HPLC on a DEAE anion-exchange column, as above. T_2 RNase cleaves RNA giving mononucleoside 3′-phosphates from internal positions and pppNp from a 5′-triphosphorylated end group.

Tobacco acid pyrophosphatase (Epicentre; Cambio, Cambridge) was used, following the manufacturer's instructions, to digest m^7G^{32}pppGmAAUACUCAAG (see above) for 30 min at 37°C, analysing products by electrophoresis on 20% polyacrylamide–7 M urea gels. [γ-^{32}P]ATP and [α-^{32}P]GTP were digested as controls, giving labelled phosphate and GMP markers respectively.

Calf intestinal phosphatase (Boehringer) digestion of 20% polyacrylamide–7 M urea gel-purified phosphorylated GmAAUACUCAAG was followed by phenol/chloroform and ether extraction of the aqueous layer. Labelling of an aliquot of the aqueous layer was carried out using [γ-^{32}P]ATP and T4 polynucleotide kinase. Products were analysed by 20% polyacrylamide–7 M urea gel electrophoresis, followed by autoradiography. A control labelling of crude non-phosphorylated GmAAUACUCAAG and phosphorylated oligonucleotide without prior phosphatase treatment with [γ-^{32}P]ATP and T4 polynucleotide kinase was done in parallel.

Maxam and Gilbert gels (13) were used for partial separation of mono- and diphosphorylated GAAUACUCAAG. 20% polyacrylamide (60:1 acrylamide:bisacrylamide) 40 cm long thin gels were used for electrophoresis in 0.5× TBE buffer for 16–20 h at 250 V. About 40 µg crude phosphorylated oligonucleotide was heated to 100°C for 2 min in formamide and loaded on the gel. Products were detected by UV shadowing.

Transcription using influenza A virus RNA polymerase *in vitro*

Transcription was carried out by standard methods (5) using influenza A virus (X-31) cores (not micrococcal nuclease treated) as a source of RNA polymerase, except that ^{32}P-labelled capped oligonucleotides (see above) were used as primers instead of ApG and no α-^{32}P-labelled nucleoside triphosphate was added to the reaction mixture. An equimolar mixture of 14 nt synthetic RNA (5′-GGCCUGCUUUUGCU-3′), mimicking the sequence at the 3′-end of virion RNA, and a 15 nt synthetic RNA (5′-AGUAGAAACAAGGCC-3′), mimicking the 5′-strand of influenza virion RNA (the so-called 'RNA fork'), was used as template (5,14). The labelled transcripts formed by incubating at 30°C for 2 h were analysed by 18% polyacrylamide gel electrophoresis in 7 M urea and the products detected by autoradiography.

RESULTS

Analysis of phosphorylation products

Four oligoribonucleotides 11–13 nt long (see Materials and Methods) were synthesized on the ABI synthesizer using standard solid phase methods and the 5′-dimethoxytrityl protecting group removed. Phosphorylation was performed (see Materials and Methods) on the protected oligonucleotides, while still attached to the solid phase support, in a separate apparatus using the phosphitylating reagent salicyl phosphorochloridite, followed by reaction with pyrophosphate and oxidation with iodine (6; Fig. 1). After deprotection and desalting on Sephadex G-25 the products were analysed by electrophoresis on 20% polyacrylamide gels in 7 M urea. In each case the major product of phosphorylation (band X) migrated slightly faster than the non-phosphorylated control oligonucleotide. About 90% of the starting material was converted to this product (results not shown).

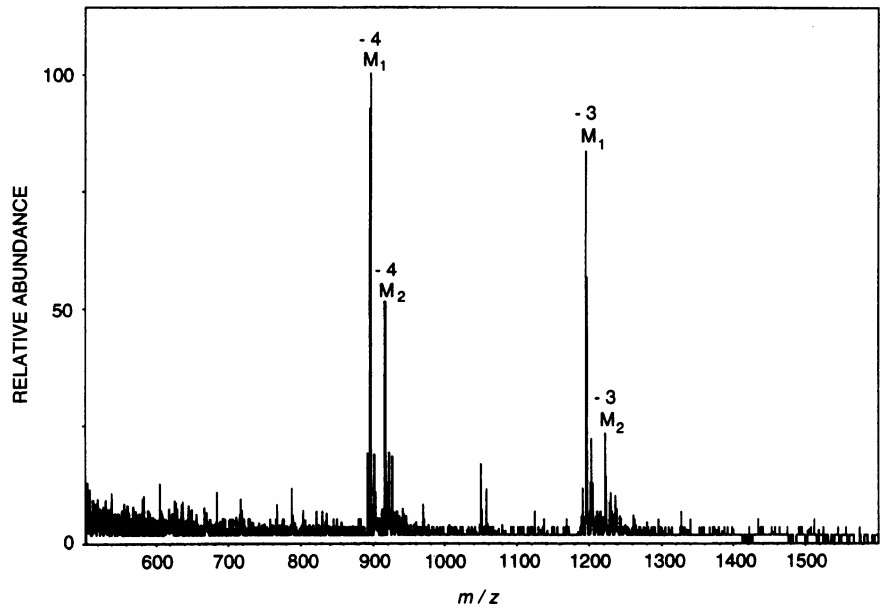

Figure 2. Electrospray mass spectrum of band X derived from phosphorylation of GmAAUACUCAAG showing ions from two components, M_1 and M_2. The net negative charge (z) on each ion is indicated, e.g. –4 denotes $(M\text{-}4H)^{4-}$, in which M represents the mass of the neutral molecule. Experimentally measured m/z values and resulting molecular masses are shown in Table 1.

Gel-purified band X (derived from phosphorylation of GmAAUACUCAAG) was initially characterized by treatment with calf intestinal phosphatase. Any free 5′-OH groups generated by such phosphatase treatment were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by gel electrophoresis (see Materials and Methods). As controls, band X and a control non-phosphorylated GmAAUACUCAAG were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase without any prior phosphatase treatment. This showed that phosphatase-treated band X gave rise to a labelled product with an electrophoretic mobility indistinguishable from 5′-^{32}P-labelled GmAAUACUCAAG. This suggested that band X had one or more phosphates added to the 5′-end of the oligonucleotide. Similar results were obtained with band X from phosphorylated U_{11}.

Figure 2 shows the electrospray mass spectrum of band X derived from the phosphorylation of GmAAUACUCAAG. This is of reasonable quality and the M_1 ions correspond to monophosphorylated oligonucleotide and the M_2 ions, in lower yield, to the diphosphorylated oligonucleotide. Similar, although slightly more 'noisy' spectra, were obtained for band X derived from phosphorylated U_{11} (results not shown). The resulting molecular masses are shown in Table 1, where it can be seen that there is excellent agreement between the found and calculated masses. No peaks corresponding to the triphosphates were detected in the mass spectrum for either oligonucleotide.

Further evidence for the presence of mono- and diphosphorylated oligonucleotides was obtained by complete enzymatic hydrolysis of phosphorylated ACACUUGCUUUUG using either P1 nuclease or T_2 RNase digestion followed by DEAE anion-exchange HPLC (see Materials and Methods). P1 nuclease gave a 5′-end product, ppA which was definitively identified at the precise elution time of marker ppA, in ~0.3 molar yield/mol oligonucleotide, as estimated from integration of the pA and ppA peaks on the chromatogram. No product was detected in the position of marker pppA suggesting that 5′-triphosphorylated products were absent or in too low yields (<10%) to be detected. The T_2 RNase products ppAp and pAp were tentatively identified, in approximately equal yields, from their elution positions, which were close, but not identical, to marker pppA and ppA.

An attempt was made to separate the mono- and diphosphorylated oligoribonucleotides by electrophoresis on non-urea 20% polyacrylamide gels run at low voltage (13; Materials and Methods). A partial separation of pGAAUACUCAAG from ppGAAUACUCAAG was observed (results not shown), the former migrating faster on the gel, as judged by its mobility compared with an internal control of 5′-^{32}P-labelled GAAUACUCAAG. However, no separation of the mono- and diphosphorylated forms of ACACUUGCUUUUG was observed.

Table 1. Molecular mass measurements by mass spectrometry

Compound	m/z (charge)	M_r found	M_r calculated
pGmAAUACUCAAG[a]	717.4 (–5), 896.7 (–4), 1195.7 (–3)	3591.0	3591.2
ppGmAAUACUCAAG[a]	916.8 (–4), 1222.5 (–3)	3670.9	3671.2
pU_{11}	482.7 (–7), 563.4 (–6), 676.3 (–5), 845.4 (–4)	3386.1	3385.9
ppU_{11}	494.0 (–7), 576.7 (–6), 692.4 (–5), 865.5 (–4)	3466.1	3465.9

[a]Mass spectrum shown in Figure 2.

Capping of phosphorylated oligoribonucleotides

Initially, labelling of phosphorylated oligoribonucleotides with [α-^{32}P]GTP and guanylyl transferase (see Materials and Methods) was done to provide further evidence for the presence of 5′-di- or triphosphorylated oligonucleotides, since this enzyme is known to accept either as a substrate (15). Capping of each of the four different crude phosphorylated oligonucleotides gave rise to a major radioactive product on a 20% polyacrylamide gel, with minor faster and slower moving products. The major capped products derived from phosphorylated GmAAUACUCAAG and phosphorylated U_{11} had electrophoretic mobilities just slower than 5′-^{32}P-labelled pGmAAUACUCAAG and pU_{11} respectively (results not shown). Further evidence for the authenticity of these capped products was the failure to observe their synthesis if the corresponding non-phosphorylated control oligonucleotides were used as substrates for guanylyl transferase. Finally, the presence of a phosphodiester linkage in the labelled capped structure m^{7}G^{32}pppGmAAUACUCAAG was confirmed by digestion with tobacco acid pyrophosphatase (see Materials and Methods). A labelled product, pm^{7}G, was identified by its similar mobility to a labelled marker, pG, on 20% polyacrylamide gel electrophoresis.

Labelled capped oligoribonucleotides are primers for influenza RNA polymerase

Previously we had confirmed earlier work (4) that m^{7}GpppGmAAUACUCAAG acted as a primer for influenza RNA polymerase *in vitro* and had investigated its priming properties with mutant templates (5). Here we extend our study to three more capped oligonucleotides allowing, firstly, a comparison of oligonucleotides differing only in the 2′-*O*-methyl group (the Cap 1 structure). Secondly, we studied priming with a capped oligonucleotide, m^{7}GpppACACUUGCUUUUG (the 5′-end of rabbit β-globin mRNA, but lacking the 6-methyl and 2′-*O*-methyl groups on the first A residue and the partial 2′-*O*-methylation of the second residue, the Cap 2 structure; 16), since β-globin mRNA has been extensively studied as a substrate in a coupled endonuclease cleavage and primed transcription reaction with influenza RNA polymerase *in vitro* (3). Thirdly, we studied the model compound m^{7}GpppU_{11}.

Figure 3 shows that equal radioactive counts of the four ^{32}P-labelled capped primers gave differing yields of transcripts on polyacrylamide gel electrophoresis with influenza RNA polymerase in response to the added model partially duplex 'RNA fork' as template (see Materials and Methods). The 2′-*O*-methylated m^{7}GpppGmAAUACUCAAG primed transcription to give a transcription product (TP) at ~10 times the yield of the products derived from the same primer lacking the 2′-*O*-methyl group (compare lanes 3 and 6). The transcript primed by m^{7}GpppGmAAUACUCAAG (lane 3) is a doublet, 23 and 24 nt long, previously characterized by partial T_1 RNase sequencing, which initiated transcription at the third nucleotide of the added template and was partially elongated at its 3′-end by the addition of a non-templated nucleotide (5). As previously noted (4,5), elongation of primers was not quantitative. A single nucleotide was added to the primer, which was not dependent on added RNA template (see Fig. 3, lanes 2, 5 and 8). This may reflect premature termination occurring on endogenous RNA template present in the influenza RNA polymerase cores. The

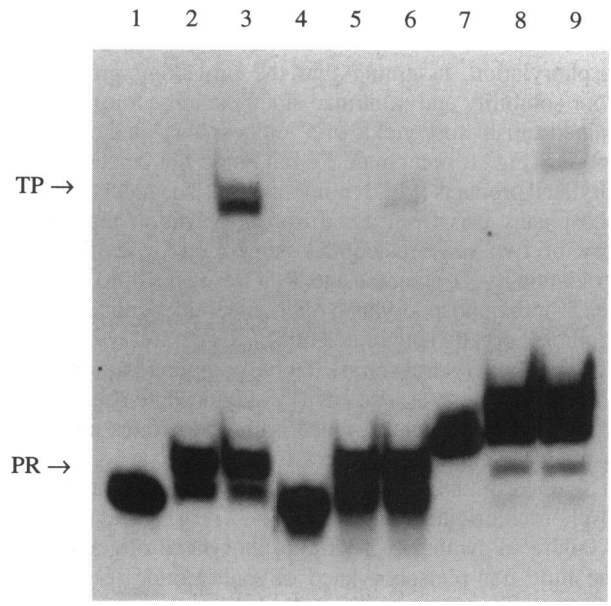

Figure 3. Capped oligoribonucleotides serve as primers for transcription by influenza virus RNA polymerase. *In vitro* transcription was carried out using viral cores as the source of RNA polymerase, a synthetic RNA template and ^{32}P-labelled capped oligoribonucleotide primers (m^{7}GpppGmAAUACUCAAG, lanes 1–3; m^{7}GpppGAAUACUCAAG, lanes 4–6; m^{7}GpppACACUUGCUUUUG, lanes 7–9) as described in Materials and Methods. Lanes 1, 4 and 7, equal radioactive amounts of primers incubated without RNA polymerase and added RNA templates; lanes 2, 5 and 8, primers elongated in the absence of RNA template, but in the presence of enzyme; lanes 3, 6 and 9, primers elongated by 14–15 nt in the presence of RNA polymerase and added RNA templates. PR, primer. The relative yields of transcription products (TP) in lanes 3, 6 and 9 were in the ratio 100:10:36, as measured by laser densitometry.

rabbit β-globin mRNA capped oligonucleotide m^{7}GpppACACUUGCUUUUG gave a triplet product (lane 9) in intermediate yield (36% of the 2′-*O*-methylated oligonucleotide) at a position on gel electrophoresis consistent with its priming on the second and third nucleotides of the added template (3). We have not been able to convincingly detect a specific product with m^{7}GpppU_{11} as a primer (results not shown).

DISCUSSION

The chemical literature on the synthesis of nucleoside triphosphates from nucleosides is very extensive, but to our knowledge such methods have not previously been applied to oligonucleotides synthesized by modern solid phase methods, except for the addition of a single 5′- or 3′-monophosphate using 2-[2-(4,4′-dimethoxytrityloxy)ethylsulphonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite (17) or 5′-monophosphate addition with 2-[*p*-nitrophenylethyl]phosphitylating reagents (18) introduced by Pfleiderer (19). Multiple phosphate additions to give di- or triphosphates are not possible with either of these reagents.

We wished to add a di- or triphosphate to an oligoribonucleotide to enable it to be subsequently capped enzymatically and then used as a primer for the influenza A virus RNA polymerase. The method of Ludwig and Eckstein (6) for the synthesis of ATP analogues from adenosine seemed ideal, especially as the reaction conditions were mild and nucleobase protection was not reported

to be required. We chose, however, to leave all protecting groups intact except for the 5′-OH group and carry out a solid phase phosphorylation, assuming that the protecting groups would favour solubility and minimize side reactions. Surprisingly, we obtained satisfactory yields of 5′-diphosphorylated oligoribonucleotides (Fig. 1, compound **5**), but could not detect 5′-triphosphorylated products (Fig. 1, compound **4**). The main evidence for diphosphates was from electrospray ionization mass spectrometry of two oligonucleotides, supported by evidence from degradation by P1 nuclease and T_2 RNase on a third. In all three cases 5′-monophosphorylated oligonucleotides contaminated the 5′-diphosphorylated oligonucleotides. Successful enzymatic capping with guanylyl transferase to give compound **6** supported the evidence for the presence of a 5′-diphosphate, but could not exclude a 5′-triphosphate, since both end groups are efficient substrates for guanylyl transferase (15).

It seemed unlikely that the initial phosphitylation by salicyl phosphorochloridite giving compound **2** (Fig. 1) was to blame for the failure to synthesize 5′-triphosphorylated oligonucleotides, since little non-phosphorylated oligonucleotide (compound 1) remained after the reaction. Probably, the pyrophosphate-catalysed double-displacement reaction giving rise to compound **3** was not optimal. ^{31}P NMR analysis showed that the tri-*n*-butylammonium pyrophosphate reagent used was impure and contained a major phosphate contaminant, as well as at least one other minor unidentified phosphate compound. Presumably these contaminants arose during the preparation of tri-*n*-butylammonium pyrophosphate from tetrasodium pyrophosphate (6). We propose that phosphate, rather than pyrophosphate, might have preferentially reacted with compound **2** (Fig. 1), giving rise to 5′-diphosphorylated oligoribonucleotides (compound **5**). We cannot exclude the possibility that the synthetic reaction conditions, or the subsequent deprotection, might have resulted in the degradation of any synthesized triphosphates, giving rise to di- or monophosphates, since triphosphates are particularly acid labile.

The 5′-diphosphorylated oligonucleotides were substrates for enzymatic capping with guanylyl transferase. Three of the four ^{32}P-labelled capped oligoribonucleotides tested primed transcription of a short RNA product using influenza A virus RNA polymerase *in vitro*. As predicted from previous work using a coupled endonuclease and priming assay (20), the 2′-*O*-methylated capped oligonucleotide m^7GpppGmAAUACUCAAG was more efficient than the same oligoribonucleotide without the 2′-*O*-methyl group. This is the first time that the effect of the 2′-*O*-methyl group has been critically tested in a priming assay, uncoupled from the effect it might have on the endonucleolytic activity of the RNA polymerase. The capped sequence m^7GpppACACUUGCUUUUG, though lacking the 6-methyl group, the 2′-*O*-methyl group on the first A residue and partial 2′-*O*-methylation of the C residue (Cap 2) of authentic rabbit β-globin mRNA, was also reasonably efficient as a primer. Our failure to detect priming with m^7GpppU_{11}, even though $m^7GpppGmC(U)_n$ (where *n* is an undefined large number) is reasonably active in a coupled endonuclease priming assay (21), may be explained by the lack of a 2′-*O*-methyl group, although other factors may be involved. Overall, our results confirm that 2′-*O*-methyl groups are important, but not essential, for priming (20). Our methods should now allow a systematic investigation of the effect of mutating all nucleotides in the primer on the efficiency of binding and transcription by the influenza RNA polymerase *in vitro*. It will also be interesting to test the effect of 2′-*O*-methylation or other 2′-*O*-alkyl groups (22) at all nucleotide residues in the oligoribonucleotide in turn or in combination.

Our chemical phosphorylation method may be ideal for large scale synthesis of short capped oligonucleotides. They are of great interest, since short capped oligonucleotides with a 3′-phosphate end group in the range 4–9 nt in length can bind to influenza virus RNA polymerase and specifically inhibit cap-dependent transcription *in vitro* (4). They are, therefore, potential specific antiviral compounds (4). It is now relatively simple to systematically investigate the optimal length, methylation state and sequence for the specific inhibition of cap-dependent transcription. Analogues (e.g. phosphorothioates or 2′-*O*-alkyl derivatives; 22) of these short primers can easily be chemically synthesized and tested as substrates for capping by guanylyl transferase. If such analogues can be capped and inhibit cap-dependent transcription by the influenza RNA polymerase *in vitro* they would be candidates for an antiviral drug, since they are known to be more resistant than unmodified oligoribonucleotides to RNase and other nuclease digestion *in vivo*.

Finally, a more detailed study of phosphate or pyrophosphate addition using this solid phase method is warranted, to establish conditions for optimal di- or triphosphate synthesis. It should also be possible to automate the reactions described here on the ABI 394 synthesizer, since no precipitates were formed in the reactions. Ultimately, a procedure for the total chemical synthesis of capped oligoribonucleotides is desirable (23). Moreover, the chemical phosphorylation described here should succeed equally well with deoxyribonucleotides, so that other biological applications for the use of 5′-di- or triphosphorylated DNA may emerge, e.g. in the antisense field, either for basic research or in the biotechnology industry.

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