Synthetic mRNA cap analogs with a modified triphosphate bridge – synthesis, applications and prospects†

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Received (in Montpellier, France) 19th January 2010, Accepted 5th March 2010
First published as an Advance Article on the web 8th April 2010
DOI: 10.1039/c0nj00041h

The cap structure was discovered at the 5′-end of the eukaryotic mRNA over three and a half decades ago. Since then, hundreds of chemically modified cap analogs have been synthesized and applied in numerous studies on the elucidation of cap-related physiological processes in the cell, and became important members of the biophosphate family. In this Perspective, we present recent developments in the synthesis of cap analogs modified within 5′,5′-triphosphate bridges and their utility for interdisciplinary studies on cap-dependent processes in gene expression and its regulation, as well as biotechnological applications and perspectives in medicine.

1. Introduction

Biological roles of the cap

Almost all eukaryotic cellular mRNAs possess a cap at their 5′-end. The cap is a very unique structure consisting of an uncommon nucleoside, 7-methylguanosine, connected *via* the 5′,5′-triphosphate bridge to the first transcribed nucleotide (Fig. 1).[1] The cap is exceptional not only because of its chemical structure, which determines its interesting physicochemical properties, but also due to numerous cellular functions during various stages of gene expression (Fig. 2), including mRNA splicing,[2] intracellular transport,[3] turnover, translation[4,5] and translational repression *via* microRNA.[6,7] Addition of the cap is the earliest event during eukaryotic mRNA synthesis. Caps are synthesized co-transcriptionally in the nucleus, when transcripts reach ~20 nt,[8,9] by an enzymatic capping complex consisting of triphosphatase, guanylyltransferase and methylase, in three steps.[1] First, the triphosphatase removes the terminal phosphate from the 5′-triphosphate of nascent oligoribonucleotide. In the next step, GMP, which is covalently bound to the guanynylyltransferase, is transferred onto the previously obtained 5′-diphosphate to create $G^{5'}$pppN on the 5′-terminus of pre-mRNA transcript. Subsequently, the distal guanosine is methylated by RNA (guanine-7)-methyltransferase, resulting in $m^7G^{5'}$pppN called cap 0. In some species, including mammals, additional methylations occur, mainly at the 2′-*O* position of the first or the first and second nucleotides of the transcript (cap 1 and cap 2, respectively).[1] Some organisms and viruses use different mechanisms to synthesize caps.[1] The capped pre-mRNA transcript interacts in the nucleus with CBC (cap binding

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† This article is part of a themed issue on Biophosphates, and is dedicated to Professor Wojciech J. Stec on the occasion of his 70th birthday.

Jacek Jemielity

Jacek Jemielity was born in 1973 in Wysokie Mazowieckie, Poland. He studied chemistry at the Faculty of Chemistry, University of Warsaw, where he received his PhD in 2002 under the supervision of Prof. Marianna Kanska. The same year he joined the group of Edward Darzynkiewicz at the Division of Biophysics, University of Warsaw, where he is currently appointed as an assistant professor. His main research interests are the synthesis, properties and applications of phosphate-modified analogs of biologically relevant nucleoside oligophosphates, including mRNA caps.

Joanna Kowalska

Joanna Kowalska studied chemistry and biology at the College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw. She earned her MSc in organic chemistry in 2006 under Dr Jacek Jemielity's supervision. The same year she began Ph.D. studies at the Division of Biophysics, University of Warsaw under Prof. Edward Darzynkiewicz's supervision, continuing research on the synthesis of mRNA cap analogs modified at the non-bridging positions of the oligophosphate bridge.

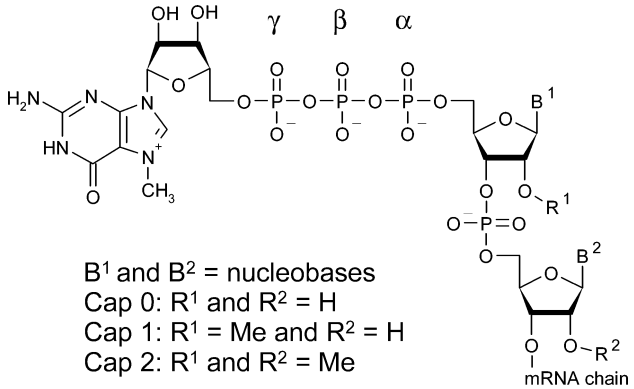


Fig. 1 A general depiction of mRNA cap structure in eukaryotes.

complex), which takes part in pre-mRNA splicing and 3′-end formation, stimulates mRNA export shuttling between nucleus and cytoplasm and participates in the first pioneer round of translation.[10,11] In the cytoplasm, mRNA associates with the eukaryotic translation initiation factor (eIF4E), which specifically recognizes the cap structure and interacts with another translation factor, eIF4G, enabling the recruitment of the small ribosomal subunit on mRNA and formation of the 48S initiation complex. eIF4E plays a central role in the translational control of gene expression.[12] The cap-eIF4E interaction is crucial and considered rate-limiting for the 48S complex formation and, hence, protein synthesis. Elevated levels of eIF4E have been found in many types of tumor cells and are connected with selective increase in the translation of mRNAs important in malignant transformation and metastasis.[13] Recent findings indicate that cap is also involved in micro-RNA-mediated translational repression, however a detailed mechanism of this process is still unclear. Finally, one of the major cap functions is in mRNA degradation.

In eukaryotes, two major routes of mRNA decay have been identified: 5′→3′ and 3′→5′ (Fig. 3). Both of them are initiated by shortening the poly(A) tail and both involve degradation of the cap structure at some stage, however, different enzymes, acting at different stages of each pathway, are responsible for its cleavage. The 5′→3′ pathway is initiated by the removal of the cap from the mRNA 5′-end, catalyzed by the Dcp2/Dcp1 complex (Dcp = Decapping protein). Dcp2, the catalytic subunit of the complex belongs to the superfamily of hydrolases containing a Nudix catalytic domain (these hydrolases use Nucleoside diphosphates linked to an X moiety as substrates).[14] Dcp2 cleaves the cap between the α and β phosphates to release m^7GDP and a 5′-phosphorylated RNA,[15] which is then digested by 5′-exonucleases.[16] The enzyme requires divalent metal cations for its activity, however, the exact catalytic mechanism is still unclear. Dcp2 does not cleave 'free' caps, but only those attached to a mRNA body of at least ~20 nt.[15]

In the 3′→5′ pathway, mRNA is first processively degraded from the 3′-end by exosome.[17] The resultant short 5′-capped (oligo)nucleotides are subsequently degraded by Decapping Scavenger enzymes (DcpS). DcpS belongs to the HIT family of pyrophosphatases,[18] which use a histidine triad to carry out hydrolysis. The enzyme cleaves the cap between the β and γ phosphates to produce m^7GMP and a downstream (oligo)-nucleotide. The crucial catalytic step is a nucleophilic attack of histidine 277 on the γ-phosphate.[19] DcpS, as opposed to Dcp2, is unable to cleave caps attached to full-length mRNAs or longer oligonucleotides.[19,20]

Synthetic mRNA cap analogs have served as invaluable tools for studying almost all of the above mentioned processes and were also employed as reagents for synthesizing capped mRNA *in vitro*. Differences in gene expression in various organisms (as well as a variety of processes and proteins involving cap structure) are the reasons why over three decades after the discovery of the cap there is still a need for novel chemically prepared analogs, which could interact more specifically with cap-related proteins, would be resistant to enzymatic hydrolysis or able to selectively inhibit or activate particular processes *etc*. This article is a personal view on the recent developments in the synthesis of cap analogs and their biological and biotechnological applications.

Anna Maria Rydzik

Anna Maria Rydzik studied chemistry at the College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw. She obtained her MSc in organic chemistry in 2009 under Dr Jacek Jemielity's supervision. She was involved in research on the synthesis of mRNA cap analogs modified at the bridging positions of tetraphosphate bridge. In the autumn 2009 she began her PhD study at Oxford University under the supervision of Christopher J. Schofield.

Edward Darzynkiewicz

Edward Darzynkiewicz received his PhD with David Shugar and carried out post-doctoral training with Aaron Shatkin. He developed an approach based on designing and synthesizing model 5′ mRNA cap analogs, including the native ones, which proved to a valuable tool in the search for molecular mechanisms of translation, splicing, RNA intracellular transport and mRNA enzymatic stability. Together with his co-workers, he proposed a new strategy of enhancing the efficiency of in vitro protein translation, based on introduction of the anti reverse cap analogs (ARCAs) into the synthetic mRNA transcripts. In 2006 he was admitted as a Howard Hughes Medical Institute International Scholar.

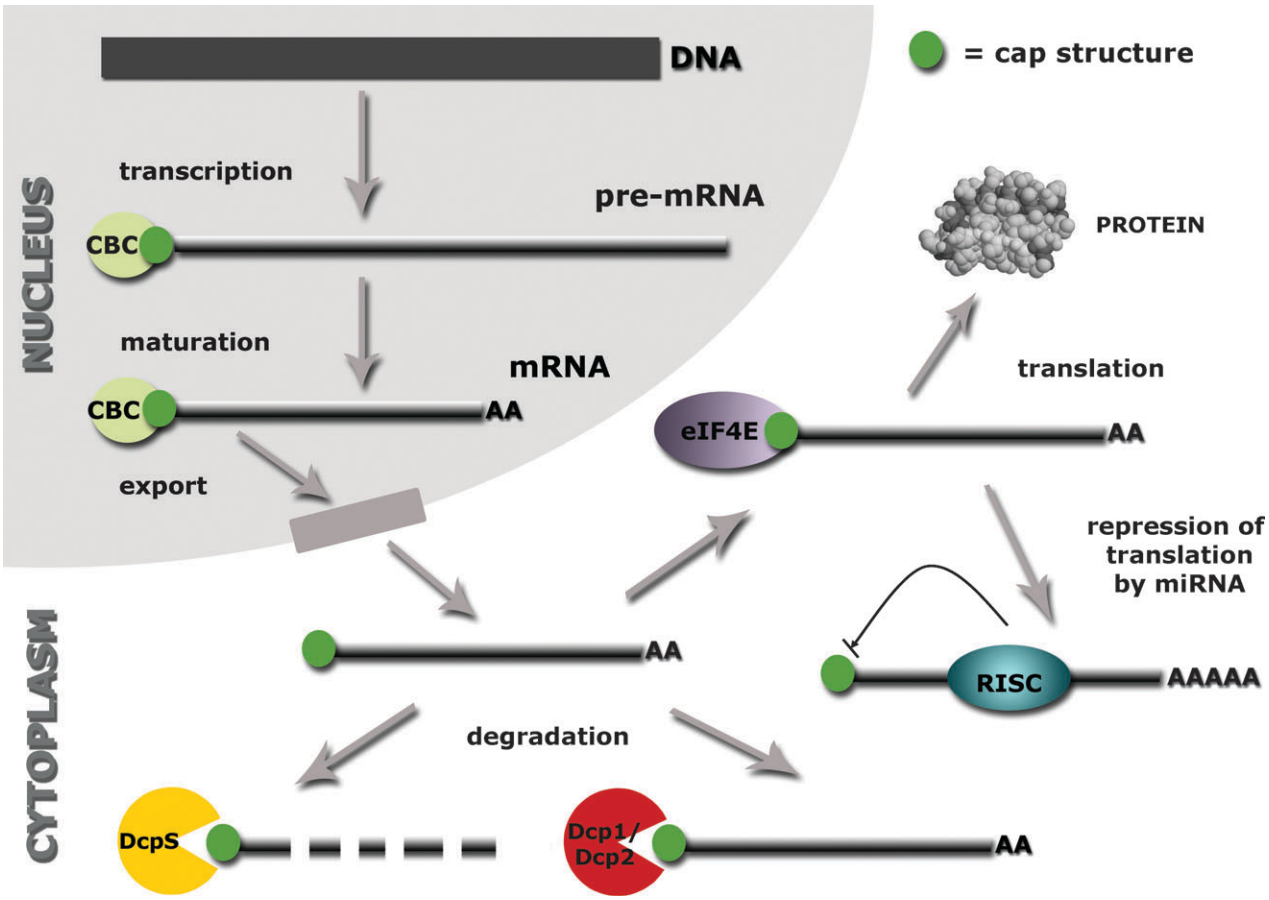


Fig. 2 Involvement of mRNA 5′-end (cap) in various biological processes in eukaryotic cells. Cap structure is synthesized on the mRNA 5′-end at a very early stage of transcription. In the nucleus pre-mature mRNAs interact with cap binding complex (CBC). This interaction stimulates efficient and accurate splicing, 3′ polyadenylation and mRNA export to cytoplasm. In the cytoplasm, CBC is displaced by a translation initiation factor eIF4E. Cap-eIF4E interaction facilitates the recruitment of a small ribosomal subunit on mRNA and the initiation of translation. Cap is also involved in mRNA degradation as well as translational repression mediated by miRNA.

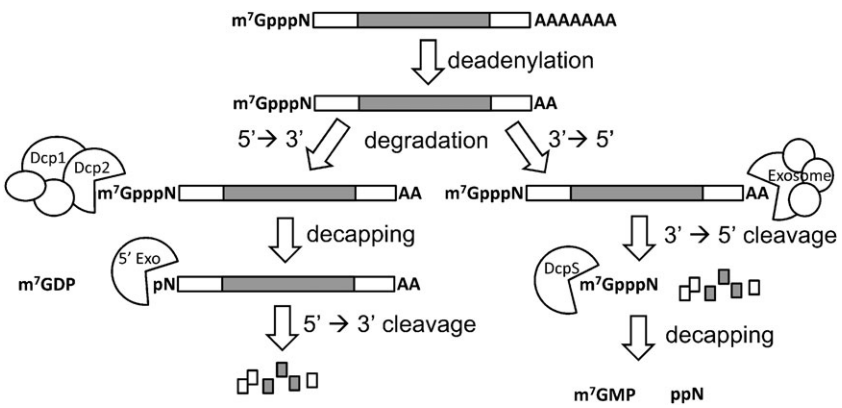


Fig. 3 mRNA degradation pathways. Both major pathways of mRNA decay are initiated by deadenylation. In the 5′→3′ pathway, the Dcp2/Dcp1 complex first removes the cap from the mRNA 5′-end cleaving it between α and β phosphates to release m^7GDP and a 5′-phosphorylated mRNA chain, which is then exposed to degradation by 5′-exonucleases. During the 3′→5′ pathway, mRNA is processively degraded from its 3′-end by the exosome and the resultant short capped RNA (oligo)nucleotides are utilized by DcpS, which cleaves the cap between the β and γ phosphates to release m^7GMP and (oligo)nucleotide 5′-diphosphate.

Special attention is paid to analogs modified within the 5′,5′-triphosphate bridge, which is currently the main interest of the authors of this article and which has proved to be an important factor in cap-protein interactions and is also the site of cleavage by decapping enzymes. Thus, such modified cap analogs are considered important tools for structural and molecular biology, biotechnology and, potentially, medicine.

2. Chemical synthesis of mRNA cap analogs

2.1 General overview

The physicochemical properties of 7-methylguanosine nucleotides were reviewed in detail earlier.[21] For the purposes of this article, it is, however, worth highlighting some of their unique properties. One of their most characteristic features is a positive charge in the 7-substituted guanine ring. Due to that charge, nucleotides containing m^7Guo take part in sandwich cation–π stacking interactions,[22] which are crucial for specific recognition of cap structures by several proteins involved in gene expression (eIF4E, CBC, DcpS, *etc.*).[19,23,24] The positively charged 7-methylguanine moiety also has a great impact on the chemistry applicable in the synthesis of cap analogs. Due to high polarity, it significantly decreases solubility of the intermediates in organic solvents. An even more limiting factor in the synthesis of cap analogs is the instability of the 7-methylguanosine-containing nucleotides in acidic, as well as basic, conditions (high susceptibility to depurination and irreversible imidazole ring opening, respectively).[21] All these features make the chemistry of cap analogs rather difficult, excluding most protecting group strategies and imposing mild conditions during synthetic steps and separation.

Generally, synthesis of nucleotides, as well as their isolation and purification, is difficult.[25] Due to their ionic nature, nucleotides are poorly soluble in organic solvents, therefore laborious conversions into organic salts are usually necessary. Nucleotides are most often used as tributylammonium, triethylammonium or pyridinium salts but even then only the most polar organic solvents are suitable reaction media for their conversions. This restriction often excludes the use of more lipophilic reactants during nucleotide synthesis and makes significant limitations to this field. The situation is even more difficult in the case of nucleotides containing 7-methylguanosine, *e.g.* mRNA cap analogs. Such nucleotides are even less soluble and more labile in acidic and basic conditions, which makes working with them even more complicated.

For purification of the nucleotides, including cap analogs, techniques of ion-exchange (IE) chromatography and reverse phase (RP) HPLC are extensively used. For separation of reaction mixtures consisting of nucleotides varying in number of phosphate moieties IE chromatography on DEAE-A25 Sephadex resin using an increasing gradient of triethylammonium bicarbonate (TEAB) in water as an eluent is particularly useful. The use of TEAB buffer is advantageous because after purification the nucleotides are isolated as triethylammonium salts and can be used after drying in the successive step of synthesis without any additional cation-exchange. Unfortunately, IE chromatography is very often insufficient to achieve the full separation of nucleotides and further purification steps are required. More efficient, but also more laborious (especially in multi-milligram scale separations), is the RP HPLC method. One of the most common eluting systems used for preparative RP HPLC is a linear gradient of methanol or acetonitrile in ammonium acetate buffer. The ammonium acetate has a very important advantage; it is easily removed from isolated nucleotides by repeated freeze-drying. However, since the compounds are obtained as ammonium salts, this method is used rather for purification of final products, otherwise, conversion of the purified nucleotides into organic salt is usually necessary.

The crucial step in the synthesis of all (di)nucleoside polyphosphates is the pyrophosphate bond formation and several widely used chemical approaches have been developed to achieve this.[25–28] In the majority of them, one nucleotide subunit is activated with a good leaving group and the second acts as a nucleophile. Several activating groups have been successfully applied for the synthesis of cap analogs,[21,29] including phenylthio,[30] 5-chloro-8-quinolyl,[31] morpholidate[32] and imidazolide.[33–35] Although different coupling methods have never been compared directly, the authors of this review believe that the *P*-imidazolides, especially used in DMF in the presence of zinc chloride excess first described by Kadokura *et al.*,[36] seem to be the most powerful activation method for the elongation of the nucleotides' phosphate chain. The pyrophosphate bond formation in anhydrous media (DMF) diminishes the extent of *P*-imidazolide hydrolysis, reducing the by-product formation, whereas the excess of zinc chloride significantly improves the solubility of reactants, serves as a Lewis acid catalyst activating imidazole as a leaving group, and acts as a template coordinating both nucleotide subunits that form the pyrophosphate bond, thus accelerating a coupling reaction. *P*-imidazolides were shown to undergo coupling with a broad spectrum of nucleophiles, including phosphate, pyrophosphate, nucleoside mono-, di, and triphosphates and, as will be described later, modified phosphate species. Fig. 4 summarizes the scope of the method applied for pyrophosphate bond formation with various substrates employing $ZnCl_2$–DMF conditions. Synthesis of *P*-imidazolides is usually achieved by one of two methods: either by the use of carbodiimidazole in DMF,[37] or by activation with imidazole using Mukaiyama and Hashimoto oxidation–reduction condensation.[38,39] In the latter method, initially 2,2′-dithiodipyridine and triphenylphosphine form a complex, which subsequently activates the terminal phosphate of the nucleotide to a oxyphosphonium derivative. Such an activated nucleotide undergoes a substitution by imidazole to form the desired *P*-imidazolide and triphenylphosphine oxide.[39] Using this method, not only nucleosides 5′-monophosphates have been efficiently activated,[33] but also nucleosides 5′-diphosphates[34,40] and even 5′-triphosphates.[40]

2.2 Syntheses of mono- and dinucleotide cap analogs with modified 5′,5′-linkage

Several modifications of the 5′,5′-triphosphate chain were introduced into mRNA cap analogs. One of them was the elongation of the triphosphate bridge to tetra-, penta- or hexaphosphate. The synthetic approach described generally above enabled synthesis of a series of dinucleoside tetra-, penta- and hexaphosphate cap analogs, as well as mononucleoside 5′-oligophosphates: 7-methylguanosine 5′-tetraphosphate and 5′-pentaphosphate,[7,40,41], which all demonstrated interesting biophysical and biological properties (see sections 3.1.2 and 3.2). It is worth emphasizing that obtaining such long polyphosphate chains required coupling of highly charged reactants, *e.g.* nucleoside triphosphate *P*-imidazolide with pyrophosphate (Fig. 4), and a only few other methods which enable extension

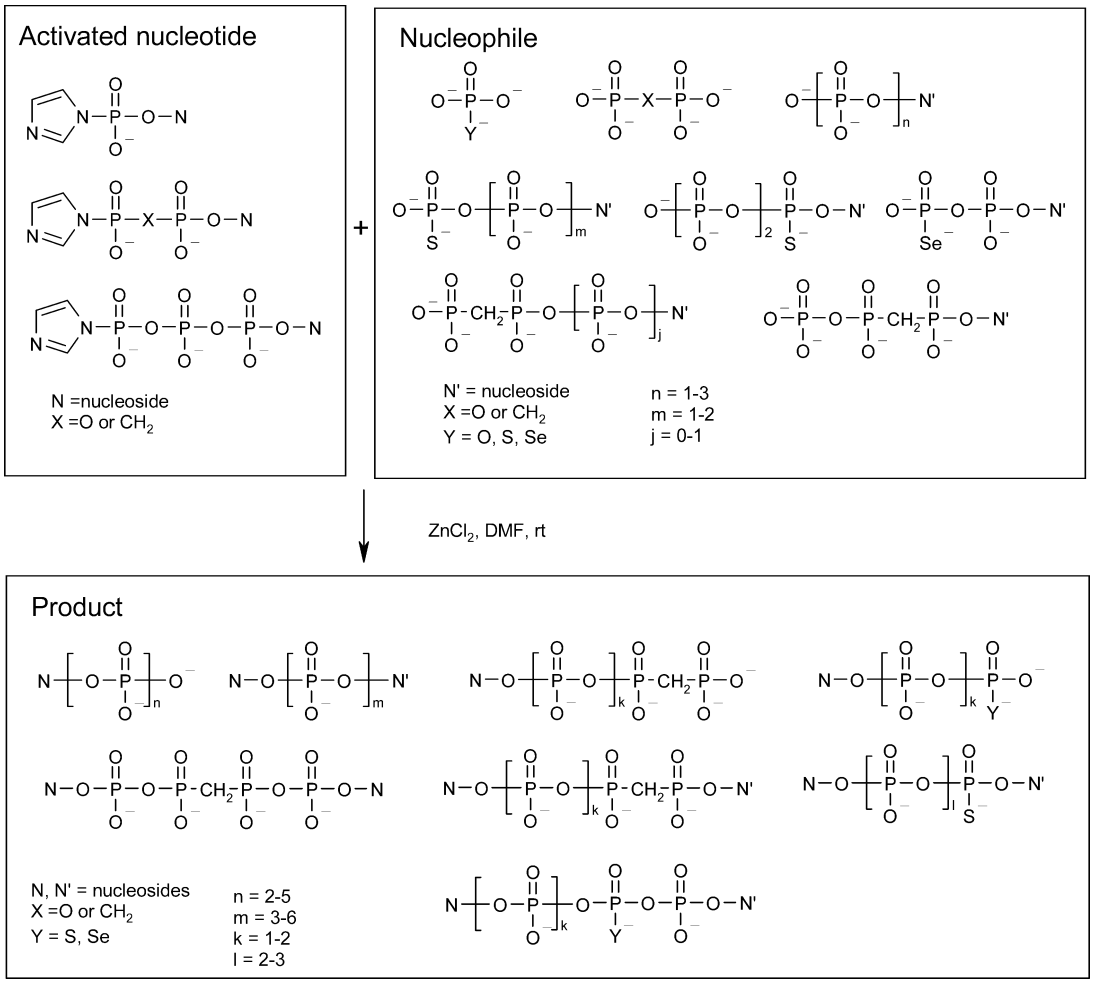


Fig. 4 A diagram summarizing syntheses of mRNA cap analogs with a modified 5′,5′-triphosphate bridge achieved *via* a $ZnCl_2$-mediated coupling reaction in DMF.

of the polyphosphate bridge beyond tetraphosphate, have been reported.

Later, the list of mRNA cap analogs available through the described synthetic frame was extended with nucleotides modified in the phosphate chain, *i.e.* in which one of the oxygen atoms of oligophosphate linkage was substituted with a different atom or group of atoms, X. The most popular modifications of the oligophosphate chain in nucleotides are presented in Fig. 5. Generally, they can be divided into two groups: those bearing oxygen-to-X substitution in either bridging or non-bridging positions. The modifications of the natural phosphate or pyrophosphate moieties in nucleotides significantly affect their chemical properties, including charge, polarity, size, hydrophobicity, basicity, and nucleophilicity, which subsequently may result in their altered biological activity, *e. g.* a change in affinity to protein factors and/or susceptibility to enzymatic hydrolysis. Hence, it is not surprising that some of these modifications have also been introduced into mRNA cap structure.

The first examples of mRNA cap analogs with a modified triphosphate bridge were analogs with a bisphosphonate modification (Table 1, m^7GppCH_2pG (**15**), m^7GpCH_2ppG (**16**) and two analogs (**17**, **18**) additionally modified with a 3′-*O*Me in the ribose of m^7Guo to assure correct incorporation during the *in vitro* transcription (anti-reverse cap analogs, ARCA, see section 3.1.1),[42] *i.e.* with one of the bridging oxygens replaced with methylene (Fig. 4). To perform an efficient synthesis of dinucleoside triphosphates with bisphosphonate modification, important requirements had to be fulfilled: (i) an efficient method for synthesis of nucleoside 5′-methylene-bis(phosphonates) and (ii) a convenient method for connection of bisphosphonate and phosphate moieties to form a triphosphate linkage with a methylene modification in the selected position. The first one was achieved by modification of Yoshikawa's 5′-phosphorylation conditions,[43] where $POCl_3$ is used as a phosphorylating agent. The straightforward, two-step, one-pot reaction with methylenebis(phosphonic dichloride) allowed us to incorporate a bisphosphonate moiety with a good yield, regioselectively into the 5′ position of the unprotected nucleoside (Scheme 1).[44] Guanosine 5′-bisphosphonate and *N*7-methyl guanosine 5′-bisphosphonate, crucial reactants for synthesis of methylene cap analogs, were obtained with 83% and 77% yields, respectively. The synthesis of a phosphate–phosphonate 5′–5′ ($ppCH_2p$) bridge was again achieved using *P*-imidazolide activated nucleotides in DMF in the presence of $ZnCl_2$, showing that nucleoside 5′-bisphosphonates can act as

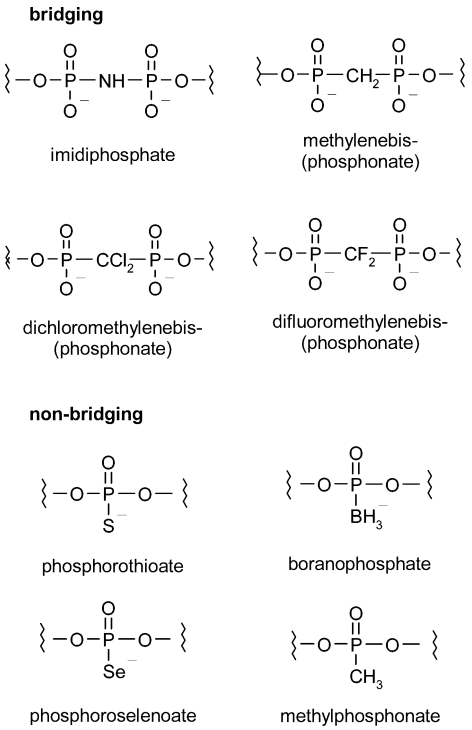


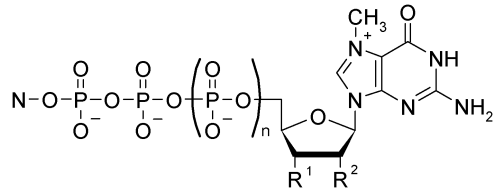
Fig. 5 Typical modifications of phosphate moieties in nucleotides.

nucleophiles in this type of reaction (Fig. 4). Earlier, triethylammonium methylenebis(phosphonate) was also employed as a nucleophile to synthesize monucleotide mRNA cap analogs m^7GppCH_2p (**26**) and $m^7GpppCH_2p$ (**27**).[45] It is noteworthy that, similarly to activated nucleoside diphosphates (ImppN), *P*-imidazolide of guanosine 5′-bisphosphonate ($ImpCH_2pG$) could be obtained,[46] however, its reactivity in coupling reactions in DMF was lower than for its diphosphate counterpart. Nonetheless, $ImpCH_2pG$ was later successfully used in coupling with triethylammonium phosphate to obtain guanosine 5′-triphosphate with methylene modification at the α,β-position.[47] This compound was an intermediate for the synthesis of one of the six cap analogs bearing a single methylene modification in the 5′,5′-tetraphosphate bridge ($m^7GpppCH_2pG$ (**20**), m^7GppCH_2ppG (**21**), m^7GpCH_2pppG (**22**), $m_2^{7,2'-O}GpppCH_2pG$ (**23**), $m_2^{7,2'-O}GppCH_2ppG$ (**24**), $m_2^{7,2'-O}GpCH_2pppG$ (**25**)).[47] Again, the crucial step in the syntheses of these tetraphosphate dinucleotides was the coupling of two nucleotide subunits to form dinucleotide 5′,5′-tetraphosphates and it was achieved with conversions from 75 to 90%. Since several routes leading to formation of tetraphosphates with single methylenebis(phosphonate) modification were possible, the most convenient method was chosen taking into account ability to activate nucleoside subunits, reactivity of reactants and effectiveness of products separation from reaction mixture. Two various synthetic pathways were studied for two of the analogs (**20**, **23**) in order to find the best coupling route.

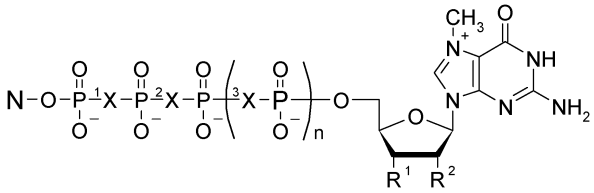
Phosphorothioates represent non-bridging modifications of the phosphate group and have also been employed for modification of mRNA cap analogs.[48] Initially, six dinucleotide cap analogs bearing a single phosphorothioate modification at either the α, β or γ position of the 5′,5′-triphosphate chain (m^7Gppp_SG (**28**), m^7Gpp_SpG (**29**), m^7Gp_SppG (**30**), $m_2^{7,2'-O}Gppp_SG$ (**31**), $m_2^{7,2'-O}Gpp_SpG$ (**32**), $m_2^{7,2'-O}Gp_SppG$ (**33**)) were synthesized. Three of them were additionally modified with methyl groups at the 2′-*O* position of 7-methylguanosine to make them anti-reverse cap analogs (ARCA). Due to the presence of *P*-stereogenic centers in the phosphorothioate moieties, each analog was obtained as a mixture of two diastereomers, D1 and D2, which were resolved by RP HPLC, providing 12 objects for biophysical and biochemical studies. The methodology applied for incorporation of the phosphorothioate moiety into the triphosphate chain depended on its position in the triphosphate bridge. Analogs modified in the α and γ positions, in which phosphorothioate group is neighbouring the nucleoside moiety, were obtained by thiophosphorylation of the appropriate nucleoside with $PSCl_3$ in trimethylphosphate (Scheme 1), followed by coupling of the isolated nucleoside 5′-thiophosphate with *P*-imidazolide of the appropriate nucleoside 5′-diphosphate in DMF in the presence of $ZnCl_2$ (Fig. 4). Formation of the mixed pyrophosphate bond was slower than in the case of unmodified phosphates and the reactions required 1–2 days to be completed. Still, the desired compounds were formed as major products. It is noteworthy that the methylation at the N7 position needed to be performed at the nucleoside stage, before the thiophosphorylation step; otherwise methylation with methyl iodide, as well as with dimethyl sulfate, preferably alkylates the sulfur atom at the phosphorothioate moiety. A slightly different route was applied for synthesis of the β-phosphate modified cap analogs. First, nucleoside 5′-(2-thiodiphosphates) were synthesized by treatment of the appropriate nucleotide *P*-imidazolide with a *ca.* 4-fold excess of thiophosphate triethylammonium salt in DMF in the presence of $ZnCl_2$.[49] Subsequently, the isolated products were again coupled with another nucleoside monophosphate *P*-imidazolide under similar conditions to form the triphosphate bridge with a phosphorothioate moiety in the central position (Fig. 4).[48] The synthesis of mRNA dinucleotide cap analogs with the phosphorothioate modification in the tetraphosphate bridge is described by Strenkowska *et al.* in the current issue of *New J. Chem.*[50] Also mononucleotide cap analogs modified with a phosphorothioate moiety have been synthesized, namely two diastereomers of m^7GTP with a sulfur atom in α position of the triphosphate chain ($m^7GTP\alpha S$, **39**).[51] The synthesis has been accomplished by a simple one-pot 5′-thiophosphorylation of 7-methylguanosine in $(MeO)_3PO$ in the presence of 2,6-lutidine, followed by an addition of tributylammonium pyrophosphate and subsequent hydrolysis of the cyclic trimetaphosphate-like intermediate (Scheme 1). For diastereomers, referred to as D1 and D2, according to their elution order during separation on the basis of ^1H NMR spectra, absolute configurations were assigned as S_P and R_P.

Another modification of the non-bridging position, closely related to phosphorothioate, is the phosphoroselenoate group. Phosphoroselenoates have recently gained much attention due to their usefulness in nucleic acid X-ray crystallography for multiwavelength anomalous diffraction (MAD) phasing techniques. The cap analog with oxygen-to-selenium substitution in β-position and the 2′-*O*-Me ARCA modification ($m_2^{7,2'-O}Gpp_{Se}pG$, **38**) was designed and synthesized on the basis of the results of biological studies with phosphorothioate

Table 1 Cap analogs modified within the 5′,5′-triphosphate bridge

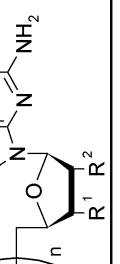


	No	Abbreviation	R^1	R^2	N	*n*	Ref. for synthesis	Ref. for application
Standards	**1**	m^7GpppG	OH	OH	Guo	1	—	—
	2	$m_2^{7,3'\text{-}O}$GpppG	CH_3	O	Guo	1	35,32	35,40,6
	3	$m_2^{7,2'\text{-}O}$GpppG	O	CH_3	Guo	1	40	40
	4	m^7GTP	OH	OH	—	1	41	41,51
	5	m^7Gp_4G	OH	OH	Guo	2	40	40,80,82
	6	$m_2^{7,3'\text{-}O}Gp_4G$	CH_3	O	Guo	2	40	40
Elongated 5′,5′-bridge	**7**	$m_2^{7,2'\text{-}O}Gp_4G$	O	CH_3	Guo	2	40	40
	8	$m_2^7$2′dGp$_4$G	O	H	Guo	2	40	40
	9	$m_2^7Gp_4$	OH	OH	—	2	41	41,82
	10	m^7Gp_5G	OH	OH	Guo	3	40	40
	11	$m_2^{7,3'\text{-}O}Gp_5G$	CH_3	O	Guo	3	40	40
	12	m^7Gp_5	OH	OH	—	3	41	41
	13	m^7Gp_6G	OH	OH	Guo	4	7	7
	14	$m_2^{7,2'\text{-}O}Gp_6G$	O	CH_3	Guo	4	7	7



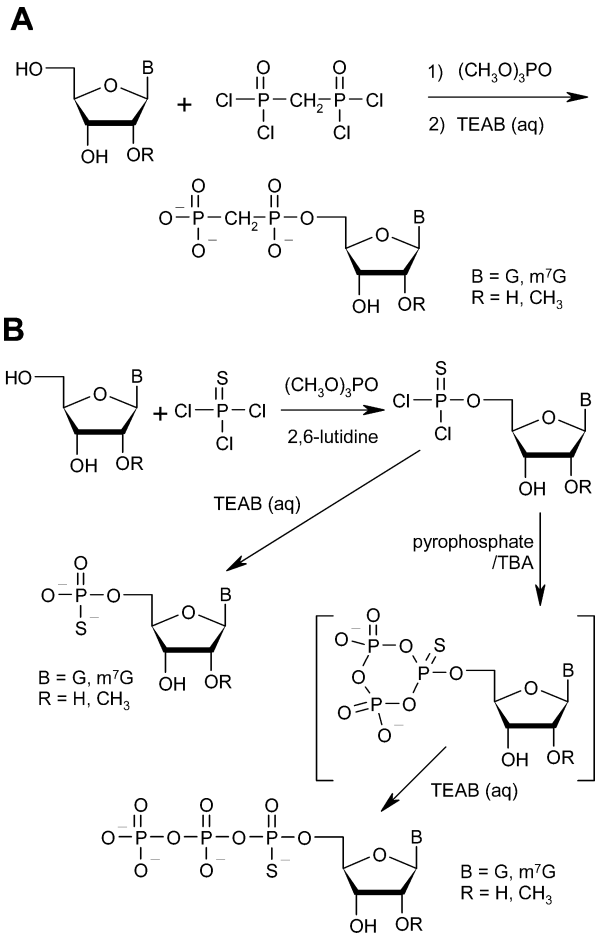
	No	Abbreviation	R^1	R^2	N	*n*	^1X	^2X	^3X	Ref. for synthesis	Ref. for application
Bridging position modified	**15**	m^7GppCH_2pG	OH	OH	Guo	0	CH_2	O	—	42	42,80
	16	m^7GpCH_2ppG	OH	OH	Guo	0	O	CH_2	—	42	42,80,74
	17	$m_2^{7,3'\text{-}O}GppCH_2pG$	OCH_3	OH	Guo	0	CH_2	O	—	42	42,64,74
	18	$m_2^{7,3'\text{-}O}GpCH_2ppG$	OCH_3	OH	Guo	0	O	CH_2	—	42	42,64,74
	19	$m_2^{7,2'\text{-}O}GppCH_2pG$	OH	OCH_3	Guo	0	CH_2	O	—	83	81,83
	20	$m^7GpppCH_2pG$	OH	OH	Guo	1	O	O	CH_2	47	47
	21	m^7GppCH_2ppG	OH	OH	Guo	1	O	CH_2	O	47	47
	22	m^7GpCH_2pppG	OH	OH	Guo	1	CH_2	O	O	47	47
	23	$m_2^{7,2'\text{-}O}GpppCH_2pG$	OH	OCH_3	Guo	1	O	O	CH_2	47	47
	24	$m_2^{7,2'\text{-}O}GppCH_2ppG$	OH	OCH_3	Guo	1	O	CH_2	O	47	47
	25	$m_2^{7,2'\text{-}O}GpCH_2pppG$	OH	OCH_3	Guo	1	CH_2	O	O	47	47
	26	m^7GppCH_2p	OH	OH	—	0	CH_2	O	—	45	—
	27	$m^7GpppCH_2p$	OH	OH	—	1	CH_2	O	—	45	—

Table 1 (*continued*)



No	Abbreviation	R^1	R^2	N	*n*	^{1}Y	^{2}Y	^{3}Y	^{4}Y	Ref. for synthesis	Ref. for application
Non-bridging position modified[a]											
28	m^7Gppp_SG	OH	OH	Guo	0	O	O	S	—	48	48
29	m^7Gpp_SpG	OH	OH	Guo	0	O	S	O	—	48	48
30	m^7Gp_SppG	OH	OH	Guo	0	S	O	O	—	48	48,74
31	$m_{2\ 7,2'\text{-}O}^{\ }GppS_G$	OH	OCH_3	Guo	0	O	O	S	—	48	48,7
32	$m_{2\ 7,2'\text{-}O}^{\ }GppS_pG$	OH	OCH_3	Guo	0	O	S	O	—	48	48,7,73
33	$m_{2\ 7,2'\text{-}O}^{\ }GpSppG$	OH	OCH_3	Guo	0	S	O	O	—	48	48,74
34	$m_{2\ 7,2'\text{-}O}^{\ }GpppS_G$	OH	OCH_3	Guo	1	O	O	O	S	50	50
35	$m_{2\ 7,2'\text{-}O}^{\ }GppS_pG$	OH	OCH_3	Guo	1	O	O	S	O	50	50
36	$m_{2\ 7,2'\text{-}O}^{\ }GpS_ppG$	OH	OCH_3	Guo	1	O	S	O	O	50	50
37	$m_{2\ 7,2'\text{-}O}^{\ }GpSpppG$	OH	OCH_3	Guo	1	S	O	O	O	50	50
38	$m_{2\ 7,2'\text{-}O}^{\ }Gpp_{Se}pG$	OH	OCH_3	Guo	0	O	Se	O	—	52	52
39	$m^7GTP\alpha S$	OH	OH	—	0	O	O	S	—	51	51

[a] Due to the presence of a *P*-stereogenic centre, all these analogs existed as two *P*-diastereomers.



Scheme 1 A. Synthesis of nucleoside 5′-methylenebis(phosphonates) B. Synthesis of nucleoside 5′-*O*-thiophosphates and 5′-*O*-(1-thiotriphosphates).

cap analogs (see sections 3.1.2 and 3.1.3).[52] The synthesis was performed essentially analogously to the synthesis of $m_2^{7,2'\text{-}O}GppSpG$ (Fig. 4), however, the first step required preparation of a very labile and prone to oxidation selenophosphate triethylammonium salt, which was obtained by treatment of tris(trimethylsilyl)phosphite with a suspension of selenium in pyridine and desilylation with methanol in the presence of triethylamine. The final products, as well as 7,2′-*O*-dimethylguanosine 5′-*O*-(2-selenodiphosphates), were the first examples of nucleotide analogs that are modified with a P_{Se} moiety at a position other than α with respect to the nucleoside.[52]

Several other analogs with various modifications of the phosphate bridge, including imidodiphosphates, dihalogenomethylenebis(phosphonates) and boranophosphates, have been recently developed and their synthesis and properties will be published soon.

3. Application of cap analogs modified within the 5′,5′-triphosphate bridge

3.1 Dinucleotide cap analogs for *in vitro* transcription

3.1.1 mRNA *in vitro* transcription: development of anti-reverse cap analogs. As already mentioned, *in vivo*, the cap is

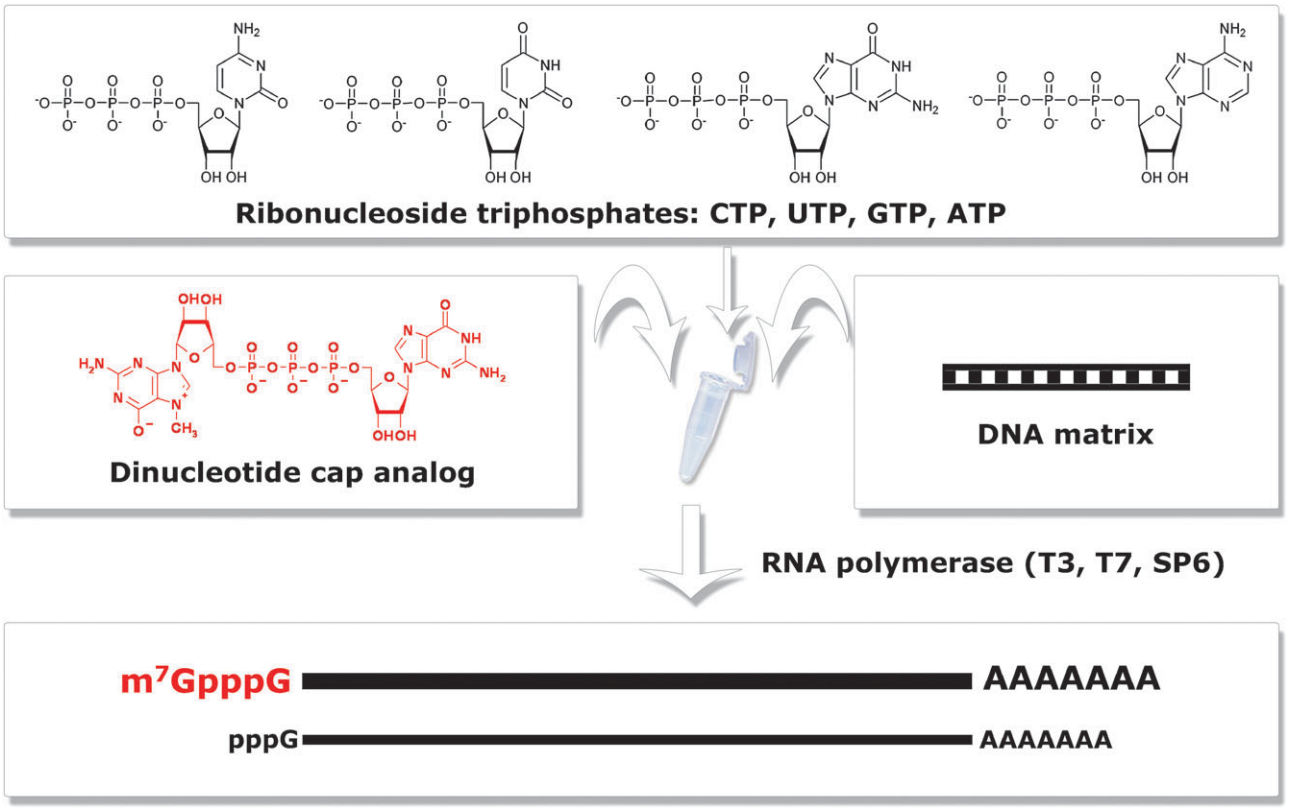


Fig. 6 A schematic representation of the synthesis of capped mRNA *via* a transcription *in vitro* in the presence of a dinucleotide mRNA cap analog. If the cap analog, such as m^7GpppG, is present in the reaction mixture in 4–10-fold molar excess in respect to GTP, the transcription is initiated from the cap analog rather than GTP, resulting mainly in capped transcripts rather than those bearing a triphosphate on their 5′-end.

added on the mRNA 5′-end at a very early stage of transcription. A usual product of *in vitro* transcription of a DNA template by an RNA polymerase in the presence of four ribonucleoside 5′-triphosphates (NTPs) is a transcript possessing a triphosphate moiety on its 5′-end. Since the cap structure plays numerous roles in mRNA metabolism, such transcripts are unsuitable for studying mRNA functions, and moreover, they are readily degraded and poorly translated in physiological systems. Capped mRNAs may be employed for expression of proteins in various eukaryotic *in vitro* translation systems or even in cultured cells, for example, in cases when the protein of interest is unstable in prokaryotic systems or requires post-translational modifications that occur only in specific cell types.

The synthesis of capped mRNA *in vitro* was first achieved over 25 years ago by transcribing a DNA template with either a bacterial or bacteriophage (T7, SP6) RNA polymerase in the presence of all four NTPs and a dinucleotide cap analog such as m^7GpppG.[53–55] These polymerases normally initiate transcription with a nucleophilic attack by the 3-OH of GTP on the α-phosphate of the next nucleoside triphosphate specified by the DNA template. If a cap analog, such as m^7GpppG, is present in the reaction mixture at a ~5 : 1 ratio to GTP, the transcription is initiated mainly by an attack of 3-OH of the cap dinucleotide rather than that of GTP, leading to the formation of capped transcripts of the form m^7GpppGpNp(Np)$_n$ (Fig. 6). However, Pasquinelli *et al.* showed that the attack can occur by the 3-OH of either the

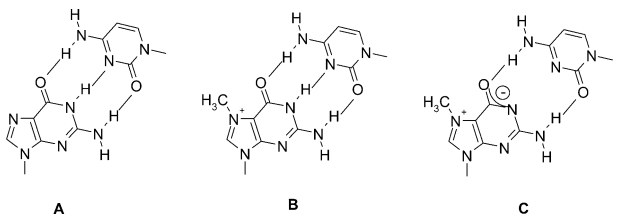


Fig. 7 Hydrogen bonding pattern between G and C (A), N1-protonated m^7G and C (B), and N1-deprotonated m^7G and C.

Guo or m^7Guo moieties of the cap dinucleotide, producing up to one half transcripts capped in a reversed orientation, *i.e.*, Gpppm^{7}GpNp(Np)$_n$.[56] This comes from the fact that the pattern of hydrogen bonding for Guo and m^7Guo is similar, so they can both effectively pair with dC in the DNA template (Fig. 7), which is the reason why the viral polymerase cannot discriminate between them. However, this is pH-dependent, since methylation at the N7-position decreases the pK_a of the N1 proton within m^7Guo to ~7.5 as compared to pK_a ~ 9 of N1 within Guo,[21] and at a pH similar to physiological pH the m^7Guo nucleotides exist as a mixture of protonated and deprotonated forms. The N1-deprotonated m^7Guo forms a less stable pair with C (Fig. 7), and, accordingly, Pasquinelli found that the portion of correctly capped transcripts was raised from 52% to 65% when the pH of the transcription reaction was increased from 6.5 to 7.5. Nonetheless, even at pH 8.4 the fraction of transcripts with reverse caps was 28%.[56]

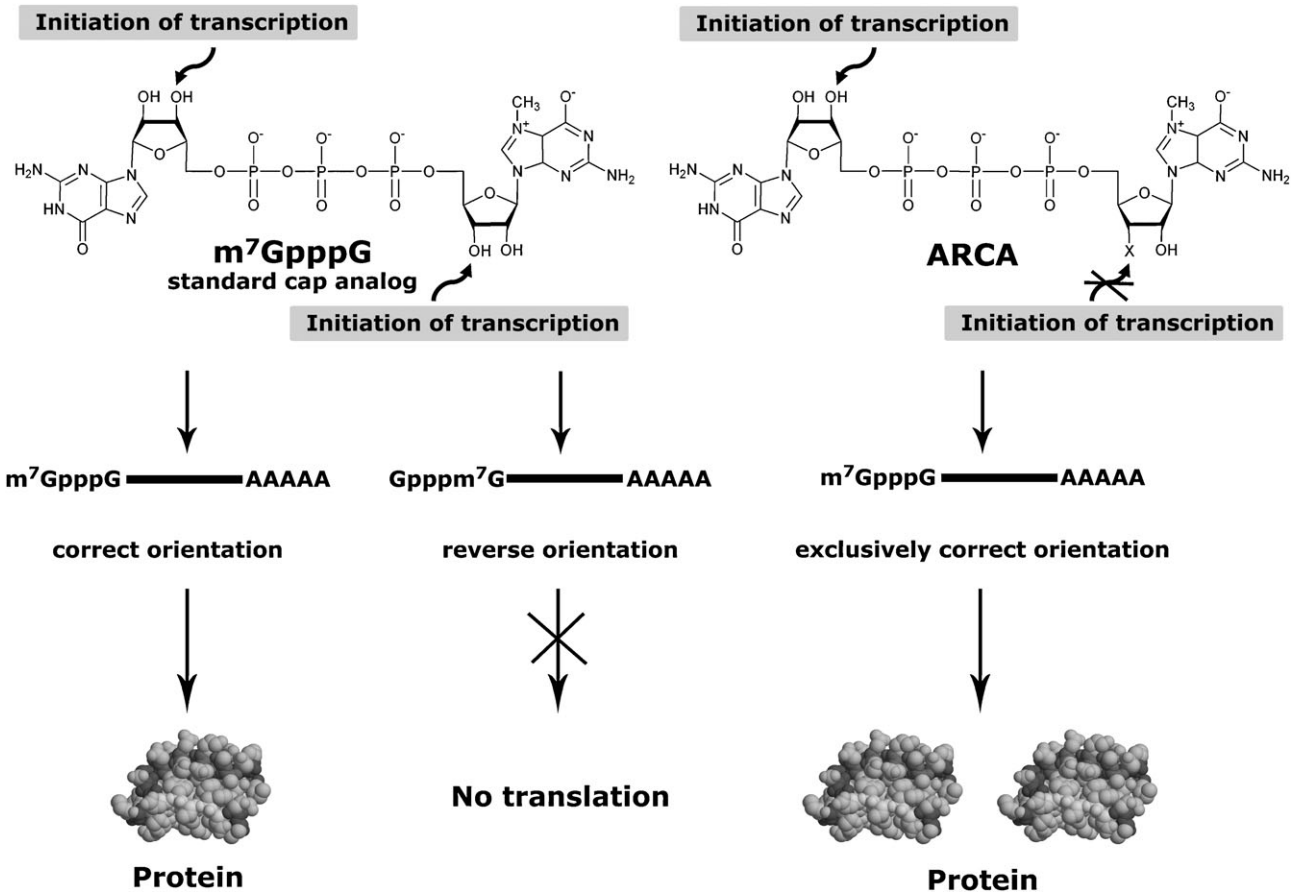


Fig. 8 The concept of anti-reverse cap analogs (ARCA). An *in vitro* transcription performed in the presence of a cap analog m^7GpppG may be initiated by an RNA polymerase from either guanosine or 7-methylguanosine to produce correctly or reversely capped mRNA, respectively. The mRNAs bearing reversely (Gpppm^{7}G..) capped structures do not behave properly, *e.g.* are poorly translated and more readily degraded. Introducing a chemical modification at the 3′-(or 2′-) position of the cap analogs' 7-methylguanosine prevents the reverse incorporation and improves both mRNA quality and translation efficiency.

The fraction of reverse-capped transcripts present in each mRNA preparation using m^7GpppG decreased its overall translational activity. Moreover, it could also influence the outcome of biological assays, as well as hamper the interpretation of results. Hence, this problem had to be solved and this was accomplished by introduction of anti-reverse cap analogs (ARCAs). In these analogs, the 3′-hydroxyl group within the m^7Guo moiety was replaced by either 3′-*O*-methyl or 3′-H modifications (Fig. 8).[35,32] Due to that modification, these analogs were incorporated into mRNA exclusively in the correct orientation, *i.e.* in which transcription is initiated by the Guo 3′-OH, and the *in vitro* translational efficiency (measured in rabbit reticulocyte lysates, RRL) of ARCA-capped mRNAs was ~2-fold higher than that of mRNAs capped with m^7GpppG.[35] Later it was shown that a substitution of 2′-OH with 2′-*O*-Me or 2′-H also ensures 100% correct orientation.[40] Surprisingly, it was found, however, that only mRNAs capped with 2′-*O*-Me-ARCAs were translated with expectedly high efficiency, whereas those capped with the 2′-deoxy ARCAs were translated only slightly more efficiently than m^7GpppG-capped mRNAs.

There is experimental evidence that ARCA substitutions do not affect the cap recognition by the translation machinery. The determined equilibrium association affinity constants (K_{AS}) for all complexes of triphosphate ARCAs with eIF4E (see section 3.2) were comparable to that of m^7GpppG.[40] Moreover, crystal structures of cap analogs bound to eIF4E reveal that both 2′- and 3′-hydroxyls are exposed to the solution and do not contribute significantly to cap recognition (Fig. 9),[23,57,58] which makes them a convenient site of potential modifications that would not affect the binding to eIF4E. Accordingly, it was recently also shown that substitutions such as 2′-OH to 2′-F, or (which are even more bulky) 2′,3′-*O*,*O*-isopropylidene or LNA, produce anti-reverse cap analogs that are translationally active.[59–61] Due to these facts, the ARCA analog $m_2{}^{7,3'\text{-}O}$GpppG became a commercially available reagent for the synthesis of capped mRNAs with high translational activity, which offers an alternative to m^7GpppG.

An alternative method for obtaining capped mRNA is the post-transcriptional capping using tri-functional Vaccinia virus capping enzyme (VCE).[62] This method has the advantages of almost 100% capping efficiency and that the cap structure obtained using this method bears no additional modifications, as compared to the "natural" cap. However, its one drawback is a high cost of such mRNA preparations, which is especially important in large scale syntheses. Both

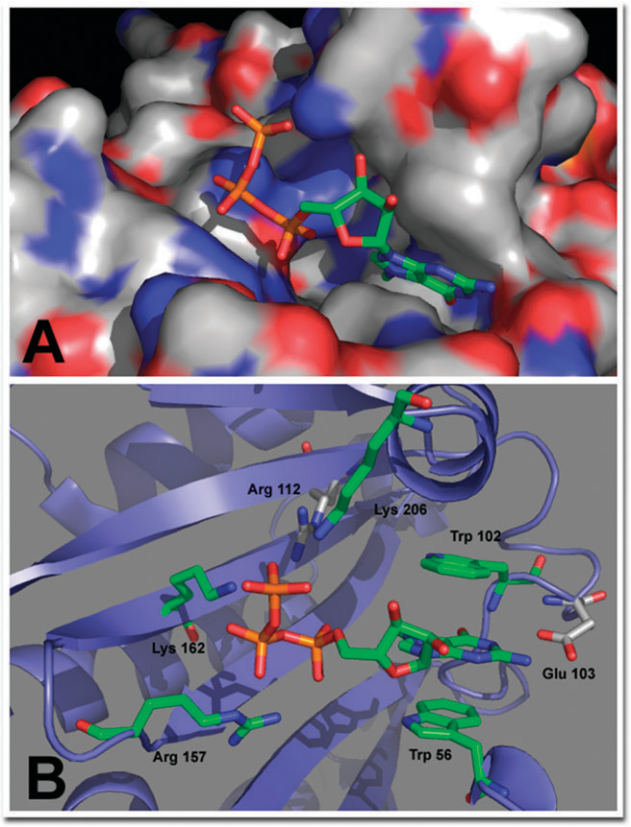


Fig. 9 Co-crystal structure of m^7GpppG bound to translation initiation factor, eIF4E (PDB entry 1L8B) A. The 2′- and 3′-hydroxyls of 7-methylguanosie are exposed to the solution and do not contribute significantly to cap binding B, aminoacids crucial for cap recognition.

capping methods, co-transcriptional (with the use of cap analogs) and post-transcriptional, result in a cap 0 structure. It is, however, possible to perform an additional methylation at the first nucleobase of the transcript (cap 1) by another enzyme, Vaccinia virus 2′-*O*-methyltransferase.[63]

An important advantage of the ARCA method over the enzymatic capping is the possibility to introduce additional chemical modifications into the 5′-end of mRNA. This creates a unique possibility to study mRNA 5′-end structure–activity relationships with respect to various cap binding proteins or mRNA-related processes. Moreover, it has already been shown that several chemical modifications may "improve" the behavior and properties of mRNAs.

3.1.2 The "second generation" ARCAs – analogs with modified 5′,5′-triphosphate bridge. The first examples of such cap analogs were those with a elongated 5′,5′-bridge.[40] It was known that the cap-eIF4E complex is stabilized not only by the stacking of 7-methylguanine between Trp-56 and Trp-102, but also by hydrogen bonds and salt bridges between the cap's negatively charged triphosphate bridge and positively charged amino acid residues in the eIF4E cap binding pocket.[23,57,58] Therefore, a series of analogs with the 5′,5′-bridge elongated to either tetra- or pentaphosphate (compound **5–8**, **10**, **11** in Table 1) were synthesized,[40] to test whether these additional residues may influence the cap recognition by eIF4E and if so, which biological consequences would be implicated. It was found that the presence of an additional, fourth phosphate moiety in the 5′,5′-bridge significantly increased the binding affinity of cap analogs (**5–8**) to eIF4E (~9–10-fold higher K_{AS} values, as compared to the triphosphate parent compounds). Furthermore, the translation efficiency measured in RRL of mRNAs bearing those tetraphosphate cap structures was slightly, but visibly (20–30%) higher than that of the corresponding triphosphate-capped mRNAs, which was explained by the fact that the increased binding affinity to eIF4E facilitates the formation of the initiation complex and thus increases the rate of ribosome recruitment on mRNA. It was also found that the addition of a fifth phosphate to the 5′,5′-bridge (compounds **10** and **11**) additionally increased the binding affinity by ~3.5–5-fold, as compared to the tetraphosphates. However, contrary to expectation, the mRNAs capped with pentaphosphate caps were translated with a lower efficiency than the mRNAs capped with tetraphosphates and with only slightly higher efficiency than those capped with triphosphates.[40] One possible explanation was that the high affinity to eIF4E inhibits the release of the protein from the complex and thus inhibits further steps of the translation process, however, other specific or non-specific protein factors may also be responsible for this effect. The tetraphosphate analogs provided the first evidence that one can "improve" the properties of an mRNA molecule by an unnatural modification of the cap structure. On the other hand, it became clear that the translation efficiency cannot be continuously improved by increasing the cap's binding affinity to eIF4E.

Hence, the next step of the search for analogs that could improve the mRNA properties were analogs that instead of having a higher affinity to translational machinery, could stabilize mRNA against enzymatic degradation, which should particularly benefit *in vivo* applications. As already mentioned in the introduction, two enzymes of complementary specificity are responsible for degradation of the cap structure in cells (Fig. 3). The Dcp2/Dcp1 complex acts in a 5′→3′ degradation pathway and is generally responsible for the removal of caps from deadenylated, but intact mRNAs, whereas DcpS acts in 3′→5′ mRNA decay, cleaving short capped RNAs released after degradation of transcripts by exosome. Therefore, it was expected that modifications protecting mRNA cap structure against the cleavage by Dcp2 rather than DcpS should influence mRNA stability *in vivo*. Taking advantage of the fact that these two enzymes cleave cap structure at different sites of the triphosphate bridge (α/β and β/γ, respectively), it was possible to design chemically modified cap analogs, which would be resistant to hydrolysis either only by Dcp2 or only by DcpS and thus, to verify this hypothesis.

Aiming at this goal, a series of four cap analogs, including two ARCAs, with the 5′,5′-triphosphate bridge modified with a methylenebis(phosphonate) moiety (*i.e.*, one of the bridging oxygens replaced by a methylene group) at either α,β-(**15**, **17**) or β,γ-positions (**16**, **18**) that were predicted to be resistant to cleavage by Dcp2/Dcp1 and DcpS, respectively, was synthesized (Table 1).[42] It was found that both analogs modified at the β,γ-position (**16**, **18**), and unexpectedly also the ARCA analog modified at the α,β-position (**17**), were resistant to DcpS *in vitro*.[42] On the other hand, oligonucleotides capped with $m_2^{7,3'-O}GppCH_2pG$ (**17**), but not $m_2^{7,3'-O}GpCH_2ppG$ (**18**)

or $m_2^{7,3'\text{-}O}$GpppG, turned out to be resistant to hydrolysis by human Dcp2 *in vitro*.[64] Then, assays were developed to measure both mRNA stability and translation efficiency inside living cells. When mRNAs capped with $m_2^{7,3'\text{-}O}$GppCH$_2$pG were transfected into a mammalian cell line, they had a longer half-life ($t_{1/2}$ = 330 min for luciferase mRNA with A_{60} tail in MM3MG cells) than mRNAs capped with $m_2^{7,3'\text{-}O}$GpppG ($t_{1/2}$ = 282 min) or m^7GpppG ($t_{1/2}$ = 156 min), which confirmed that blocking the cleavage of the cap by the enzyme involved in the 5′→3′ degradation pathway can stabilize mRNA *in vivo*.[64] In contrast, mRNA capped with $m_2^{7,3'\text{-}O}$GpCH$_2$ppG was even less stable ($t_{1/2}$ = 180 min) than $m_2^{7,3'\text{-}O}$GpppG-capped mRNA. Unfortunately, substituting one of the bridging oxygens with a CH$_2$ group coincidentally decreased the affinity of new analogs to eIF4E by a factor ~2, as compared to $m_2^{7,3'\text{-}O}$GpppG (K_{AS} = 4.65 ± 0.03 and 4.41 ± 0.02 μM^{-1} for $m_2^{7,3'\text{-}O}$GpCH$_2$ppG and $m_2^{7,3'\text{-}O}$GppCH$_2$pG, respectively, as compared to 7.4 ± 0.1 μM^{-1} for $m_2^{7,3'\text{-}O}$GpppG). Hence, the translation efficiency of mRNAs bearing those analogs was highly diminished both *in vitro* and *in vivo*, only slightly exceeding the efficiency of m^7GpppG-capped mRNA.[64] This effect decreased the overall protein yield from these mRNAs and could also explain the decreased stability of $m_2^{7,3'\text{-}O}$GpCH$_2$ppG-capped mRNA, as some experimental results suggested that inability to bind eIF4E may increase the rate of mRNA degradation.[64] Thus, although these analogs provided valuable insight into the interdependence of translation and mRNA 3′→5′ and 5′→3′ degradations, they proved to be rather unsuitable as reagents for synthesis of mRNAs for high yield protein translation.

These studies again emphasized the importance of the triphosphate backbone for the interaction with eIF4E. The loss of affinity to eIF4E caused by oxygen-to-methylene substitution is probably a result of incapability of the CH$_2$ group to form an H-bond important for the stability of the complex. On the other hand, these results, combined with the previous data for analogs with elongated the 5′,5′-bridge, emphasized a qualitative, but not quantitative, correlation between the binding affinity of cap analogs to eIF4E and the translation efficiency of mRNAs bearing these analogs.

The above findings provided a good rationale for exploring other possible phosphate chain modifications to find those protecting mRNA against degradation, but not decreasing affinity to eIF4E. Some of the phosphate mimics, most closely resembling natural phosphates in terms of stereochemistry, electronic structure, charge distribution and pK_a, are phosphorothioates. Therefore, a series of cap analogs (**31–33**) modified with a phosphorothioate moiety (*i.e.* with one of the non-bridging oxygens replaced by sulfur) at either the α-, β- or γ-position and at the 2′-*O* position of the m^7Guo to make them ARCAs, was synthesized and employed for biochemical studies. Due to the substitution at the non-bridging oxygen, each synthesized analog existed as a mixture of two *P*-diastereomers. Each pair of diastereomers was resolved by reverse-phase HPLC (and referred to as D1 and D2, due to their elution order) and studied further as diastereomerically pure samples. The first encouraging finding was that all of these new S-ARCAs, in contrast to CH$_2$-modified cap analogs, had affinity to eIF4E that was not lower than the parent ARCA, and for some of them the affinity was up to 4-fold higher.[48] At that point, it was unknown whether a single, non-bridging phosphate modification of the 5′,5′-linkage was sufficient to protect mRNA from decapping and if so, which position, α, β (or least likely γ), could protect from degradation by Dcp2, since the exact mechanism of the hydrolysis performed by the enzyme was, and still remains, unknown. It was found that only the oligonucleotides capped with $m_2^{7,2'\text{-}O}$GppSpG (D2) were resistant to hydrolysis by recombinant human Dcp2 *in vitro*, whereas those capped with $m_2^{7,2'\text{-}O}$GppSpG (D1) and both isomers of $m_2^{7,2'\text{-}O}$GpppSG were hydrolyzed, but slower than transcripts modified with $m_2^{7,2'\text{-}O}$GpppG or $m_2^{7,2'\text{-}O}$GpSppG.[65] The susceptibility of mRNAs to decapping by Dcp2, similarly to those bearing caps modified with the CH$_2$ group, correlated with their half-life *in vivo*. mRNAs capped with $m_2^{7,2'\text{-}O}$GppSpG (D2) had a $t_{1/2}$ of 257 min in cultured HC11 mammary epithelial cells, as compared to 155 min for $m_2^{7,2'\text{-}O}$GpppG-capped mRNA and 86 min for m^7GpppG-capped mRNA. mRNAs capped with $m_2^{7,2'\text{-}O}$GppSpG (D1) had a $t_{1/2}$ of 185 min, whereas the $t_{1/2}$ of mRNAs capped with four other analogs were in the range of 140–170 min.[65] Unexpectedly and contrary to the case of the CH$_2$-modified analogs, mRNAs translation efficiency *in vivo* correlated well with their $t_{1/2}$, not with the binding affinities to eIF4E. Luciferase mRNAs capped with $m_2^{7,2'\text{-}O}$GppSpG (D2) and $m_2^{7,2'\text{-}O}$GppSpG (D1) were translated 2.4-fold and 1.3-fold, respectively, more efficiently in HC11 cells than those capped with $m_2^{7,2'\text{-}O}$GpppG (and 5.8-fold and 2.8-fold more efficiently than those capped with m^7GpppG). For mRNAs capped with other S-ARCAs, the translation efficiency was comparable to that of $m_2^{7,2'\text{-}O}$GpppG-capped mRNA.[65]

Combining the results of the described studies allowed a general conclusion to be drawn that two features of the cap structure influence the overall yield of the protein produced from the mRNA, particularly *in vitro*. The first one is the affinity of the cap to eIF4E and the second is its susceptibility to enzymatic decapping. However, it appears that for different cap structures different factors may be determinant. For CH$_2$-ARCAs, the mRNA translation efficiencies *in vivo* correlated with K_{AS} for cap-eIF4E complexes, not with mRNA $t_{1/2}$, whereas for S-ARCAs the correlation was quite opposite. Thus, it is possible that when the level of cap binding by eIF4E becomes sufficiently high (*e.g.* at least at the level of the unmodified cap structure), some other steps in protein synthesis initiation become rate limiting. However, for the tetraphosphate ARCAs, for which the binding affinity to eIF4E was even higher than for S-ARCAs, the translation efficiencies *in vitro* were 20–30% higher. Hence, it cannot be excluded that the differences between K_{AS} of S-ARCAs were too small to observe the correlation with translation efficiencies. It is also possible that different factors may be crucial for determining the overall protein yield *in vitro* (in RRL) and *in vivo*. mRNA recruitment may be abnormally fast in the RRL system because initiation factors are present at higher levels than in other cells. Summarizing, our current knowledge about the initiation of protein synthesis and mRNA decapping does not explain these observations. Therefore, to address these issues, new compounds have been synthesized.[47,50] Two series of tetraphosphate ARCAs, one modified with a single

methylene group at different positions (**23–25**) and the second modified with a single phosphorothioate group at different positions (**34–37**), may help to answer the question whether the gain of binding affinity resulting from the presence of additional phosphate can compensate the loss due to the presence of the methylene group and improve the translational efficiency of mRNA *in vivo* and whether combining the phosphorothioate modification with an additional phosphate may superimpose and additionally be beneficial for mRNA translation *in vivo*. Although the results of translation *in vitro* are promising, to fully understand these processes detailed studies *in vivo* are necessary and are currently in progress.

3.1.3 Future prospects: capped mRNAs in gene therapy. The greater yield of protein due to combining higher translational efficiency with longer $t_{1/2}$ of mRNA should be especially beneficial for applications that utilize RNA transfection as an alternative way of gene delivery. Such potential mRNA-based gene therapy has attracted much interest over the last years. It is considered particularly promising for applications such as anticancer or anti-viral immunotherapy, but applications in other areas of gene therapy are also emerging.[66,67] mRNA as a gene therapy tool does not share the drawbacks that are often pointed out for DNA-based therapeutics, *e.g.*:

- the use of mRNA excludes the possibility of integration into the host genome and thus makes insertional mutagenesis impossible
- the effect is transient and treatment could be repeatable, hence, a long-term uncontrolled expression of a transgene is avoided
- mRNA is expressed effectively also in non-dividing cells
- mRNA is expressed in the cytoplasm, which can be reached more easily than nucleus and by use of non-viral delivery tools
- in contrast to DNA-viral vectors, non-specific immune system response is less likely.

One of the most intensively exploited mRNA therapeutic applications is immunization against autologous tumors with autologous dendritic cells (DCs) transfected with mRNAs-encoding tumor-associated antigens.[68] Dendritic cells can be either isolated from the patient, transfected with RNA *ex vivo* and then used to immunize the patient, but there are also model studies on immunization *in vivo* by a direct mRNA injection.[66,67,69] One of significant difficulties that have to be overcome before clinical application of mRNA-based therapeutics is the lability of mRNA. Several methods have been developed to stabilize mRNA and increase the efficiency and duration of protein expression in DCs (both *ex vivo* and *in vivo*). These include introduction of stabilizing sequences into 5′ and 3′ UTRs,[69,70] incorporation of pseudouridine instead of uridine,[71] and elongation of the poly(A) tail.[70] Also the cap structure was identified as a factor influencing the mRNA stability and translation in DCs. It was reported that the use of ARCA-capped mRNA alone or together with a long poly(A) tract enhanced protein expression in cultured mouse DCs by 20- and 700-fold, respectively.[72] Therefore, the potential of ARCAs modified within the 5′,5′-triphosphate bridge has also been recently investigated in this context and proved to be very encouraging. Briefly, it has been found that $m_2^{7,2'\text{-}O}GppSpG$ (D1)-capped mRNA was markedly more stable and more efficiently translated in immature DCs than $m_2^{7,2'\text{-}O}GppSpG$ (D2) or $m_2^{7,3'\text{-}O}GpppG$-capped mRNAs. The immunization of mice by intranodal injection of naked $m_2^{7,2'\text{-}O}GppSpG$ (D1)-capped mRNA gave a significantly higher level of reporter protein expression *in vivo* as well as a more potent specific immune response, as compared to other tested RNAs, making this ARCA analog a preferred choice for DCs transfection applications.[73]

3.2 Other applications of mRNA cap analogs

Apart from the application for synthesis of capped mRNA, cap analogs modified in the phosphate bridge have found several other applications. Among those applications are structural studies on cap binding proteins involved in mRNA metabolism. As was mentioned in the Introduction, during the initiation of translation, the cap is recognized specifically by eukaryotic Initiation Factor 4E (eIF4E), which stimulates the recruitment of ribosomes to mRNA. Crystallographic and NMR studies allowed identification of the amino acids essential for eIF4E–cap interaction in the eIF4E cap binding site.[23,57,58] The structural basis for the specificity of the cap structure recognition are the so called sandwich cation–π stacking interactions of 7-methylguanine between tryptophan indol rings (Trp56 and Trp102) and electrostatic or H-bond interactions (direct or water-mediated) between the negatively charged phosphate chain and positively charged side chains of lysine and arginine residues (Arg112, Arg157, and Lys162) (Fig. 9). The cap–eIF4E interaction was also investigated in biophysical studies by a fluorescence titration method based on the quenching of intrinsic Trp fluorescence in the eIF4E cap binding site upon cap analog binding.[23] These studies allowed a very detailed characterization of structural features of the cap, which are crucial for the interaction with eIF4E and also allowed determination of the modifications, which can additionally stabilize the complex. As already mentioned, the triphosphate bridge was identified as one of these key structural features of the mRNA cap. First, the equilibrium association constants (K_{AS} values) determined for eIF4E complexes with both mono- and dinucleotide cap analogs with elongated 5′,5′-polyphosphate chains were much higher than for the standard reference analog, m^7GpppG,[40,41] underlining the importance of hydrophilic/electrostatic interactions in cap recognition.[40,41] Later, it was found that replacing either of the bridging oxygens by a methylene group decreases the K_{AS} value ~1.5–2-fold and this change has notable biological implications (*e.g.* low affinity to translational machinery or small potency to inhibit cap-dependent translation – see below). On the other hand, the oxygen-to-sulfur substitutions generally stabilized the complex and at selected positions resulted in up to 4-fold higher K_{AS} values.

The fluorescence titration method was subsequently also used to study the DcpS decapping enzyme.[42,74] In agreement with the fact that DcpS carries out its hydrolysis by nucleophilic attack on the cap's γ-phosphate,[19] all cap analogs modified at the bridging β,γ- or non-bridging γ-position of the 5′,5′-triphosphate bridge proved to be resistant to DcpS and for these analogs K_{AS} for DcpS-cap complexes has been

Table 2 Comparison of the equilibrium binding affinity constants (K_{AS}) of selected cap analogs for two cap-specific proteins, eIF4E and DcpS

Cap analog	K_{AS}(A) cap-eIF4E (μM^{-1})	K_{AS}(B) cap-DcpS (μM^{-1})	R K(B)/K(A)
$m_2^{7,2'-O}GpCH_2pppG$	44.8 ± 2.0[a]	13.6 ± 0.9[a]	0.3
$m_2^{7,2'-O}Gp_SppG$ (D2)	12.9 ± 0.4[b]	14.8 ± 0.6[c]	1
m^7Gp_SppG (D1)	30.8 ± 0.2[b]	146 ± 6[c]	5
m^7GpppG	9.4 ± 0.4[b]	N.D.	N.D. (≫1)
m^7GpCH_2ppG	6.3 ± 0.3[a]	234 ± 14[a]	37

[a] Data from ref. 47. [b] Data from ref. 48. [c] Data from ref. 74.

determined. Interestingly, the influence of particular modifications on the binding affinity was rather opposite to that observed for eIF4E. Hence, the phosphorothioate group decreased the affinity, as compared to the methylenebis(phosphonate) group. However, in the case of DcpS, it is difficult to determine which affinity is closer to the "natural" cap, since the unmodified analog, m^7GpppG, is a substrate for the enzyme and K_{AS} cannot be determined by fluorescence titration. However, some semi-quantitative experimental data indicate that the affinity of DcpS to m^7GpppG is significantly higher than that of eIF4E.[20] Another difference between the cap recognition by DcpS and eIF4E was the effect of the ribose methylations at 2′-*O* or 3′-*O*-positions (ARCA modifications). As mentioned earlier, for eIF4E the ARCA modifications have virtually no influence on binding affinity.[40] In contrast, for DcpS both ARCA substitutions result in a significant decrease in K_{AS}.[42,74] Combinations of ARCA substitutions and phosphate modifications resulted in a wide range of compounds ranging in their binding affinities to DcpS and eIF4E by more than two orders of magnitude. To emphasize these differences, factor R was introduced, defined as $R = K_{AS(DcpS)}/K_{AS(eIF4E)}$. Some representative examples are given in Table 2. These findings can be useful for development of selective inhibitors directed against either DcpS or eIF4E. Analogs with high R values should efficiently compete with natural caps for DcpS, but not eIF4E and opposedly for compounds with low R values.

Finding selective inhibitors of eIF4E is important due to its involvement in oncogenesis. Studies demonstrated that the reduction of the eIF4E level or repressing its activity may inhibit the growth of tumor cells and induce apoptosis.[75,76] One of the successful antitumor therapeutic approaches based on antisense oligonucleotide directed against eIF4E is currently under clinical trials.[77] Numerous synthetic cap analogs were shown to inhibit cap-dependent translation *in vitro* by competing with mRNA for eIF4E.[40,78] The inhibitory potency correlated generally with the binding affinity to eIF4E.[40,41] Although it was proved that cap analogs can be efficient inhibitors of translation *in vitro*, their activity *in vivo* has not yet been demonstrated.

The major limitation of nucleotides application *in vivo* is their instability in cellular conditions. Also mRNA cap analogs would be targeted by enzymatic hydrolysis both extra- and intracellularly. In this context, resistance of cap analogs to the Decapping Scavenger is a particularly important factor, as this pyrophosphatase is specific for these types of compounds. Recently,[51] mononucleotide cap analogs bearing the phosphorothioate group at the position neighbouring to m^7Guo, two diastereomers of $m^7GTP\alpha S$ referred to as D1 and D2, were shown to be efficient and stable inhibitors of translation. The more potent of the $m^7GTP\alpha S$ diastereomers, (D1), inhibited cap-dependent translation in rabbit reticulocyte lysate (RRL) 8-fold more efficiently than m^7GTP (**4**) and 15-fold more efficiently than m^7GpppG (**1**). Both diastereomers were also significantly more stable in RRL than unmodified ones. After incubation of cap analogs in RRL, the unmodified analogs **1** and **4** lost a significant part of their activity, whereas $m^7GTP\alpha S$ D1 and D2 retained their inhibitory potency.[51] Surprisingly, this inhibitory activity after incubation correlates with resistance against DcpS, which was also confirmed in further studies on modified cap analogs.[47] In methylene-modified tetraphosphate-series analogs m^7GpCH_2pppG (**22**) and m^7GppCH_2ppG (**21**) were found to be resistant toward DcpS, whereas $m^7GpppCH_2pG$ (**20**) was hydrolysable. All three showed good inhibitory properties in a RRL system, but after the incubation, the hydrolysable analog **20** lost its activity, whereas activities of compounds **21** and **22** were retained on the same levels.

Cap analogs as inhibitors of DcpS activity may also become important, as DcpS has been recently identified as a therapeutic agent for spinal muscular atrophy.[79]

As was mentioned above, a modification of the oligophosphate bridge within cap analogs seems to be particularly useful for enzymatic studies, including characterization of newly identified enzymes,[80] investigation of substrate specificity of enzymes,[74] and search for inhibitors of enzymatic activity.[42,47,48]

Stabilization of the mRNA 5′-end with a non-hydrolysable cap analog also created unique possibilities for structural and molecular biology. NMR studies of the Dcp2 enzyme, which is involved in a critical step of several eukaryotic mRNA decay pathways, are an example of a stabilized mRNA 5′-end application in structural biology.[81] Application of $m_2^{7,2'-O}GppCH_2pG$ (**19**) for transcripts synthesis resulted in obtaining mRNA resistant towards decapping mediated by Dcp2/Dcp1 enzymatic complex, thus a stable mRNA-enzyme macromolecular complex was formed but the catalytic step was inhibited. Subjecting such a stable complex to NMR spectroscopy allowed determination of the interactions of the yeast Dcp2 with the cap and RNA body. It was shown that Dcp2 forms a channel for RNA, which intersects the catalytic and regulatory Dcp1-binding domains. The studies indicated that the interaction with cap is rather weak, yet specific, and required binding of the RNA body to a dynamic interface of the enzyme.[81] In other studies,[64] the 5′→3′ mRNA degradation pathway was inhibited using mRNA cap analog **17** resistant to Dcp2/Dcp1 to test relative contributions of the two predominant mRNA degradation pathways (5′→3′ *vs.* 3′→5′). The results indicated that both the pathways make major contributions to the overall degradation.

Phosphate-modified cap analogs were also employed in studies on the molecular mechanism of how miRNAs repress mRNA translation.[7] A library of cap analogs was tested in *Drosophila* embryos to investigate how miR2 inhibits translation initiation. Two of the tested analogs **13** and **31**, modified in phosphate backbone , strongly augmented the repression of translation of miRNA regulated transcripts, indicating the

primary role of cap structure in miRNA-mediated inhibition of translation.

4. Perspectives

In this article, we have reviewed recent advances in synthesis of mRNA cap analogs, focusing on modifications of the triphosphate bridge as an important factor contributing to the biological properties of the cap. Although the idea of such modifications of cap analogs is quite new, various applications of analogs modified in this way have already been shown. Additionally, synthetic routes that allow one to obtain these new compounds contributed to the development of some new approaches for formation of modified oligophosphate bridge, which could be implemented in the synthesis of other biologically relevant (di)nucleoside oligophosphates. The authors of this article are excited about the possibilities created by the modifications of the cap structure within the triphosphate linkage, which allow to investigate both well established as well as new roles of the cap, which are still being revealed 35 years after its discovery. These possibilities include, among others: ability to either inhibit or activate some cellular processes, ability to stabilize macromolecular complexes and to investigate structure-function relationship for various cap binding proteins. In our opinion, one of the most spectacular examples of the cap's importance is the fact that a single-atom modification within its structure can significantly influence the biological properties (*e.g.* translation efficiency and stability) of the whole mRNA molecule consisting of more than 80 000 atoms. The first results are very promising and encourage further exploration of this field: some new mRNA cap modifications and some new applications are under investigation and will emerge soon. The authors' deep conviction and hope is that it will bring us closer to the success of RNA-based gene therapy.

The cap structure has numerous important functions in gene expression. Until now, the 5′,5′-bridge modified cap analogs have been applied to study only few of them, which does not reflect their broad scope. Thus, studies on mRNA degradation, initiation of translation and repression of translation are still in progress. In our opinion, the new analogs should be particularly useful for studying cap binding proteins that contain divalent metal ions in the binding site, *e.g.* Dcp2 pyrophosphatase. As it is known from previous studies on enzyme mechanisms, various phosphate modifications can dramatically change the affinity of nucleotides to metal cations in ligand binding sites.

In some species, cap structures bear additional and very unique modifications, *e.g.* the so called Cap 4 in *Leishmania* or trimethylguanosine cap in nematodes. Moreover, caps are present not only at the mRNA 5′-end but also in other RNAs synthesized by RNA Polymerase II, *e.g.* some snRNAs. Hence, there are many other synthetic targets and potential applications of modified cap analogs. Finally, many eukaryotic viruses have also developed different processes to provide 5′ caps on their mRNAs (capping or cap-snatching), and they have cap-binding proteins, *e.g.* VP39 from Vaccinia virus. These phenomena could be used to inhibit the viral activity. Taking into account the above-mentioned variety of possible applications, it can be said that the time of phosphate-modified cap analogs has not finished, but rather has just started.

Acknowledgements

We are grateful to all former and present members of the Laboratory of Gene Expression, Division of Biophysics, University of Warsaw, and our coworkers, especially Robert E. Rhoads (Louisiana State University, USA), for their excellent research contributing to our understanding of mRNA cap structure chemistry and biology reviewed in this article. Financial support from the Polish Ministry of Science and Higher Education (No. NN301 243 436), National Science Support Project 2008–2010 (No. PBZMNiSW-07/I/2007), and Howard Hughes Medical Institute (to E.D.; No. 55005 604) is gratefully acknowledged.

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