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Robert E. Rhoads *Editor*

Synthetic mRNA

Production, Introduction Into Cells, and Physiological Consequences

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Cover illustration: Bioluminescence image of a living mouse after intranodal injection with synthetic mRNA encoding firefly luciferase (as described by Kreiter *et al.* in Chapter 11)

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Preface

The dream of altering genetic expression to alleviate human disease states—gene therapy—has existed from the dawn of the molecular biology era. Early attempts were made with both DNA and RNA as carriers of genetic information, but the inherent instability of RNA compared with DNA attracted by far the larger share of researchers, vectors, protocols, and success stories. Yet DNA, regardless of whether it is introduced by viral or nonviral modalities, carries the potential of integration into the host genome at unintended sites, which can lead to unwelcome and permanent consequences for the patient. Also, there are some outcomes of gene therapy that are best achieved by transient rather than permanent introduction of new genetic information, e.g., lineage conversion of cell fates, genome editing, and activation of the immune system against pathogens or cancer. For these applications, RNA, particularly mRNA, can be preferable to DNA. The past decade has witnessed new discoveries on how exogenous mRNA activates the host cell's innate immune response to shut down protein synthesis and destroy the RNA, but importantly, there have also been new discoveries on how this can be managed by modifying the structure of mRNA. Progress has also been made on methods to introduce mRNA into cells, to stabilize it in the cell, and to enhance its translational efficiency. New synthetic techniques have been developed that allow for structural features to be built into mRNA which provide investigational tools, such as fluorescence emission, click chemistry, and photochemical crosslinking. Finally, there have been significant advances in the use of synthetic mRNA for protein replacement therapy, immunotherapy against cancer and infectious diseases, creation of pluripotent stem cells from somatic cells, and genome editing. The chapters in this volume present detailed laboratory protocols for (1) synthesis of mRNA with favorable properties, (2) introduction of the synthetic mRNA into a variety of cell types by a variety of techniques, and (3) use of synthetic mRNA to achieve a range of physiological outcomes.

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Part I

Introduction

Chapter 1

Synthetic mRNA: Production, Introduction into Cells, and Physiological Consequences

Robert E. Rhoads

Abstract

Recent advances have made it possible to synthesize mRNA in vitro that is relatively stable when introduced into mammalian cells, has a diminished ability to activate the innate immune response against exogenous (virus-like) RNA, and can be efficiently translated into protein. Synthetic methods have also been developed to produce mRNA with unique investigational properties such as photo-cross-linking, fluorescence emission, and attachment of ligands through click chemistry. Synthetic mRNA has been proven effective in numerous applications beneficial for human health such as immunizing patients against cancer and infections diseases, alleviating diseases by restoring deficient proteins, converting somatic cells to pluripotent stem cells to use in regenerative medicine therapies, and engineering the genome by making specific alterations in DNA. This introductory chapter provides background information relevant to the following 20 chapters of this volume that present protocols for these applications of synthetic mRNA.

Key words Cap analogs, Immunotherapy, mRNA stability, Nucleoporation, Electroporation, Cationic lipids, Innate immunity, Protein expression, Poly(A), Translational efficiency

1 Introduction

The vision of gene therapy—correcting medical defects by introducing genetic information into the patient—was present at the dawn of the molecular biology age. In fact, some of the researchers involved in deciphering of the genetic code [1] were among the first to begin investigating the possibility of gene therapy [2, 3]. An initial question was what form of genetic material should be used to implement gene therapy. Even though support has come from many quarters that an "RNA world" preceded the current "DNA world" [4], RNA is vastly less stable to hydrolysis than DNA, both in the laboratory and the cell, due the presence of a 2′-OH group in RNA that makes formation of an internal phosphodiester intermediate possible. Therefore, DNA was used for initial attempts at gene therapy [3], and today DNA-based vectors for gene therapy are actively under development, including

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nonreplicating adenoviruses, adeno-associated viruses, lentiviruses, retroviruses (the latter being RNA viruses that go through a DNA phase), and others. Yet DNA-based gene therapy protocols have sometimes resulted in serious adverse effects in patients [5], and DNA, regardless of whether it enters the cell by a viral or nonviral route, has the potential of integrating into the host genome, causing insertional mutagenesis and activating oncogenes.

The use of mRNA as a genetic vector is theoretically attractive because mRNA does not integrate into the genome, is immediately available for translation to make protein, and provides a transient signal, a feature that is desirable for some applications. The demonstration that mRNA could, in fact, function in this capacity came in 1990, not long after the initial attempts at DNA-based gene therapy, when Wolff et al. injected naked mRNA encoding chloramphenicol acetyl transferase into the skeletal muscle of mice and observed specific protein expression [6]. This was followed in 1992 by a study in which injection of a naked, synthetic mRNA encoding arginine vasopressin into the hypothalami of Brattleboro rats cured the chronic diabetes insipidus suffered by this strain [7]. Then in 1993, Martinon et al. showed that subcutaneous injection of liposome-encapsidated mRNA encoding the influenza virus nucleoprotein induced anti-influenza cytotoxic T lymphocytes [8]. But despite these early successes, there was little progress in the use of mRNA for therapeutic purposes for a decade while development of DNA-based strategies progressed rapidly. Yet today, mRNA-based approaches are being taken to solve a wide range of biomedical problems—from vaccination against cancer and infectious diseases to generation of induced pluripotent stem cells (iPSCs) to genome editing. This change in the landscape of gene therapy is due to a number of discoveries, insights, and technical advances, discussed briefly below and more extensively in the 20 following chapters of this volume.

This introductory chapter is intended to provide background information to place the protocol chapters in context. It is not a complete treatment of the subject of synthetic mRNA and mRNA-based therapeutics. For that, the reader is referred to several recent, comprehensive, and scholarly reviews [9–12]. Also, there are RNA-based methods of altering genetic expression that do not involve mRNA, e.g., Sendai virus and miRNA, that are not covered in this volume but are reviewed elsewhere [13].

2 Basic Methodology for the In Vitro Synthesis of mRNA

2.1 The RNA Chain

mRNA fulfills its role of being a transient carrier of genetic information by being unstable in the cell, and this instability extends to the laboratory as well, where special precautions are needed to prevent mRNA hydrolysis by omnipresent ribonucleases. Yet it

is possible to synthesize RNA in the laboratory both chemically and enzymatically. For chemical synthesis, repetitive yields of >99 % are needed to make RNA chains of only 100 nt, but physiological mRNAs are typically 1000 - 10,000 nt [14]. For this reason, highly processive bacterial and bacteriophage RNA polymerases such as T7 [15] and SP6 [16] must be used. A plasmid is typically used as transcription template for the 5′-untranslated region (UTR), coding region, 3′-UTR, and sometimes poly(A) tract, with transcription driven by a promoter specific for the RNA polymerase.

2.2 The Cap

An essential feature for eukaryotic mRNA is the cap, a 5′-terminal 7-methylguanosine attached by a 5′–5′ triphosphate bridge to the RNA [17]. The cap is needed for both high translational efficiency and high stability in the cell, and these two factors separately increase the yield of protein synthesized. However, RNAs made by bacterial and bacteriophage polymerases are not capped unless extra steps are taken. Two methods are widely used to cap mRNAs, and both are employed in the chapters of the current volume. In the first method, capping is posttranscriptional and is accomplished by a multifunctional enzyme from vaccinia virus that first incorporates GTP into the RNA chain in a 5′-to-5′ triphosphate linkage, with concomitant production of P_i and PP_i, followed by 7-methylation of the 5′-terminal guanosine with S-adenosylmethionine [16, 18]. In the second method, a cap dinucleotide of the form m^7GpppG is added along with the other four NTPs during RNA synthesis [19–21]. The RNA polymerase incorporates m^7GpppG in the place of GTP at the 5′-end of the RNA. To increase the percentage of capping, the concentration of GTP is lowered relative to the other NTPs and the concentration of cap dinucleotide is raised. Each method has advantages and disadvantages. The capping efficiency is greater with the vaccinia method, but the cap dinucleotide method is co-transcriptional, so capping and in vitro transcription are performed in a single step. Furthermore, the cap dinucleotide method permits incorporation of chemically modified caps that confer additional properties to synthetic mRNAs (see below). In the current volume, several chapters use the vaccinia method (Boros et al., Holstein et al., Koh et al., and Domashevskiy et al.) and several use the cap dinucleotide method (Dannull and Nair, Borch et al., Idorn et al., Benteyn et al., Kreiter et al., Bire et al., Su et al., Devi and Nath, and Kowalska et al.).

2.3 The Poly(A) Tract

Another critical element for translational efficiency [22] and stability [23] of mRNA is the 3′-terminal poly(A) tract. Synthetic mRNA containing a poly(A) tract may be generated either by including a poly(dT) tract in the plasmid transcribed by the RNA polymerase or by posttranscriptional polyadenylation with a poly (A) polymerase [24]. Again, there are advantages and disadvantages of each method. Longer poly(A) tracts can be obtained and

modified nucleoside residues can be incorporated with poly(A) polymerase, but transcription from a DNA template yields RNA with a defined poly(A) tract length and constitutes a one-step procedure.

3 Improving the Stability and Translational Efficiency of Synthetic mRNA

The inherent lability of mRNA is a major obstacle to using synthetic mRNA for altering cellular function because a short half-life of mRNA in the cell means a short window for protein expression. However, some natural mRNAs can have half-lives measured in days rather than minutes, e.g., ovalbumin [25], globin [26], casein [27], and histone [28], and stabilization of these mRNAs is regulated by factors such as the cell cycle and hormonal signaling. Understanding the mechanisms that govern stability has allowed various investigators over the years to introduce structural changes to synthetic mRNA to improve its stability. The chapter by Su et al. in this volume describes synthesis of mRNA that exhibits the regulated stability properties of histone mRNA. Poor translational efficiency also diminishes protein expression, leading investigators to optimize this parameter in synthetic mRNA as well. Interestingly, the two most important determinants of stability and translational efficiency, the cap and poly(A) tract, act synergistically [29–33]. Structural modifications to increase stability and translational efficiency can be divided into the various domains of mRNA: cap, UTRs, coding region, poly(A) tract, and 3′-end.

3.1 The Cap

3.1.1 ARCAs

A disadvantage of the cap dinucleotide method not mentioned above is that m^7GpppG is incorporated in either orientation [34]. This is because the α-phosphate of the first nucleotide of the growing RNA chain can be attacked by the 3′-OH of either the guanosine or 7-methylguanosine moiety of m^7GpppG. A normal linkage, m^7GpppGpG…, results from attack by the 3′-OH of the guanosine moiety, but a reversed linkage, Gpppm^{7}GpG…, results from attack by the 3′-OH of the 7-methylguanosine moiety. Thus, roughly half of the caps are incorporated backwards. The manner in which caps interact with proteins involved in mRNA processing, translation, and turnover [35–37] indicates that mRNAs with the structure Gpppm^{7}GpG… would not be recognized as being capped. This problem has been solved by synthesis of a variety of cap dinucleotides in which either the 2′ or 3′ position of the 7-methylguanosine moiety is modified to prevent incorporation in the reverse orientation [38–43]. The use of these "anti-reverse cap analogs" (ARCAs) allows synthesis of mRNAs with superior translational properties both in vitro [38, 39, 44] and after introduction into various cell types [33, 44, 45]. ARCAs also permit the unambiguous location of modifications within the cap with respect

to the mRNA body and, hence, within the active sites of cap-binding proteins involved in splicing, translation, and turnover of mRNA. For example, a modification of the phosphate moiety of the triphosphate bridge that is proximal to the 7-methylguanosine moiety would always be distal to the mRNA body, provided there is an ARCA modification to prevent reverse incorporation.

3.1.2 Caps that Are Resistant to Decapping Enzymes

M-ARCAs—mRNA is degraded principally by 3′-to-5′ and 5′-to-3′ pathways [46, 47], the latter requiring prior decapping. There are two types of decapping enzymes in eukaryotic cells, DcpS and the Dcp1-Dcp2 complex. DcpS is a scavenger decapping enzyme and acts on the short capped oligonucleotides remaining after 3′-to-5′ hydrolysis by the exosome, cleaving the cap between the β- and γ-phosphates to produce m^7GMP [48]. Dcp1 and Dcp2 are the central components of an RNA-dependent decapping complex that requires an RNA body of at least 25 nt and cleaves between the α- and β-phosphate moieties to produce m^7GDP [49–53]. Substitution of the β–γ bridging O atom with CH_2 in the cap dinucleotide to produce $m_2{}^{7,3'\text{-}O}GpCH_2ppG$ prevents cleavage of this bond in vitro by DcpS [54]. Substitution of the α-β bridging O atom with CH_2 in the cap dinucleotide to produce $m_2{}^{7,3'\text{-}O}GppCH_2pG$ prevents cleavage of this bond in vitro by Dcp2 and stabilizes the mRNA to intracellular degradation in cultured cells [44].

S-ARCAs—Unfortunately, mRNAs capped with $m_2{}^{7,3'\text{-}O}GppCH_2pG$ are poorly translated in cultured cells because $m_2{}^{7,3'\text{-}O}GppCH_2pG$ has a lower affinity for the translational cap-binding protein eIF4E than the corresponding unmodified ARCA [44]. However, substituting S for the non-bridging O on the β-phosphate to make the phosphorothioate cap dinucleotide $m_2{}^{7,2'\text{-}O}Gpp_SpG$ allows one to synthesize mRNA that is resistant to in vitro decapping by Dcp2, is more stable in cultured cells, and is translated efficiently in cultured cells [55, 56]. One diastereomer of $m_2{}^{7,2'\text{-}O}Gpp_SpG$ produces mRNA that is actually translated *more* efficiently in cultured mouse mammary epithelial cells than the control (non-phosphorothioate) ARCA [55]. Injection of luciferase mRNA containing a phosphorothioate-modified cap into the lymph nodes of mice produces more luciferase than the same mRNA capped with a standard ARCA, and injection of an antigen-encoding mRNA containing a phosphorothioate-modified cap induces a more potent immune response than the control mRNA [57].

B-ARCAs—Despite the fact that mRNA capped with the most cleavage-resistant phosphorothioate analog is resistant to cleavage by Dcp2 in vitro, it is still slowly cleaved [58]. Substitution of the β non-bridging O with BH_3 to form the two-headed borano analog, $m^7Gpp_{BH3}pm^7G$ (BTH), yields mRNA that is more resistant to cleavage by Dcp2 in vitro than mRNA capped with the phosphorothioate analog, yet it is still translated as efficiently in HeLa cells [58].

3.1.3 Other Modified Cap Analogs

Fluorescent Cap Analogs for Biophysical Studies—All phases of mRNA metabolism require the specific interaction of mRNA with proteins: synthesis by RNA polymerases, splicing, modification of nucleoside residues, transport from nucleus to cytosol, targeting to specific intracellular sites of translation, translation, movement into and out of P bodies, and finally degradation. Frequently the portion of mRNA recognized by these proteins is the cap, since this is a structural feature unique to mRNA. (The caps of snRNAs are *N*-trimethylated and not recognized by most proteins involved in translation). Fluorescence spectroscopy is widely used to study protein-RNA interactions. It is therefore useful to synthesize mRNAs containing a fluorophore in or near the cap structure. In one such study, mRNA labeled at the ribose of the 7-methylguanosine moiety with either anthraniloyl or *N*-methylanthraniloyl moieties was synthesized by the one-step cap-dinucleotide method described above [59]. In a different study, anthraniloyl labeled mRNA was synthesized by the two-step vaccinia method [60]. The chapter by Domashevskiy et al. in this volume provides a detailed protocol for the latter synthesis.

Fluorophosphate-containing Caps for NMR Studies—Compounds containing ^{19}F are useful for NMR studies. Baranowski et al. [61] prepared a series of cap analogs and synthetic mRNAs in which there is an O-to-F substitution at the γ-position of the triphosphate chain and have used them to study two proteins that interact with the mRNA cap, DcpS and eIF4E.

Caps Amenable to Click Chemistry—Click chemistry describes a powerful collection of synthetic chemical reactions characterized by high yield and rapid delivery of a single product under ambient conditions, often involving azide–alkyne additions [62]. Addition of a 4-vinlybenzyl moiety to the N2 position of 7-methylguanosine in the mRNA cap allows one to create a "clickable" recipient for introducing reporter groups to mRNA to study various steps of mRNA metabolism and intracellular location [63]. The chapter by Holstein et al. in this volume provides a detailed protocol for this synthesis.

6-Thioguanosine-containing Caps for Photo-cross-linking Studies—Another way to study the interaction of mRNAs with other macromolecules is through photo-cross-linking experiments. Nowakowska et al. created cap analogs in which the guanosine moiety of m^7GpppG is changed to 6-thioguanosine to create a photo-cross-linking reagent [64]. The cap dinucleotide is synthesized as an ARCA to ensure correct orientation and is incorporated into mRNA co-transcriptionally. The chapter by Kowalska et al. in this volume provides a detailed protocol for this synthesis.

3.2 The 5′- and 3′-UTR

The UTRs of mRNA can have a profound effect on both stability and translational efficiency [65–68]. Various investigators have compared 5′- and 3′-UTRs to achieve maximum expression of proteins encoded by synthetic mRNA introduced into mammalian cells. Karikó et al. [69] found that replacing the 5′- and 3′-UTRs of urokinase-type plasminogen activator receptor mRNA with those of *Xenopus* β-globin mRNA produced a ~10-fold increase in expression of the encoded protein when the mRNA was introduced by cationic lipid-mediated delivery into several cultured mammalian cell lines. Holtkamp et al. [70] found that sequential α-globin 3′-UTRs present in a head-to-tail orientation between the coding region and the poly(A) tract each independently enhanced stability and translational efficiency of mRNA when introduced into immature human dendritic cells (DCs) by electroporation. Although delivered by a DNA-based vector, mRNA encoding the hepatitis B virus surface antigen caused the highest level of secretion of IFN-γ by splenocytes isolated from mice when it contained the 3′-UTR of rabbit β-globin mRNA [71].

3.3 The Coding Region

Two of the chapters in the current volume utilize codon-optimized mRNAs (Boros et al. and Bire et al.). Expression of proteins from synthetic mRNA can be diminished if the mRNA contains rare codons or rate-limiting regulatory sequences. Redesign of the mRNA by using synonymous but more frequently used codons can increase the rate of translation and hence, translational yield [72]. Also, recognition of the termination codon is influenced by adjacent nucleotide sequences [73]. An interesting observation was that a "silent" polymorphism in the MDR1 gene encoding P-glycoprotein is not really silent since it changes the activity and substrate specificity of the protein, suggesting that a rare codon can affect the timing of co-translational folding and insertion of proteins into the plasma membrane [74]. Mauro and Chappelle [75] have recently reviewed potential drawback of codon optimization such as this, including increased immunogenicity of proteins made from codon-optimized mRNA, and have cautioned that codon optimization may not provide the best strategy for increasing protein production.

3.4 The Poly(A) Tract

It was recognized early that the 3′-terminal poly(A) tract of mRNA is a stability factor and that deadenylation precedes 3′-to-5′ degradation of mRNA [23]. Poly(A) is also a strong stimulator of translational initiation due the fact that translation factor eIF4G associates with both the cap-binding protein eIF4E and the poly(A)-binding protein Pab1 [76]. As a result, the cap structure and the poly(A) tract synergize and direct ribosome entry to the 5′-end to drive efficient translation [77, 78]. This is referred to as the "closed loop" model for translation [79–81]. Even though mRNAs emerge from the nucleus with a long (200–300 nt) poly(A) tract, it must be

maintained in the cytosol for continued stability and translation, and mechanisms exist for cytoplasmic polyadenylation [82, 83]. The positive correlation between poly(A) length and protein expression was shown in a particularly dramatic case involving ARCA-capped mRNA used to transfect an immortalized mouse DC line, where increasing the poly(A) length from 64 to 100 nt increased protein expression by 35-fold [33]. In another study where immature human DCs were transfected with ARCA-capped mRNAs, an optimal poly(A) length of 120 nt was determined [70]. Brown et al. [84] identified a triple-helical element in the 3′-end of some RNAs that prevents deadenylation. When added to an intronless β-globin reporter RNA it increased intracellular levels. The chapter by Brown and Steitz in the current volume presents a protocol for construction and use of this stabilizing element.

3.5 The 3′-End

Structures other than poly(A) at the 3′-end can stabilize mRNA. The best understood mRNAs of this type encode the canonical replicative histones [85]. Synthesis of these histones (H2A, H2B, H3, and H4) occurs only when DNA is being synthesized and is regulated primarily by changes in mRNA levels, which increase 35-fold as cells enter S-phase. At the end of S-phase or when DNA synthesis is inhibited, histone mRNAs are rapidly degraded. The regulated stabilization of these mRNAs is conferred by a conserved 25- to 26-nt stem-loop (SL) at the 3′-end instead of poly(A). The SL is sufficient to confer regulated stability to a reporter mRNA that is introduced into mammalian cells by electroporation [86], and such a system can be used to gain insight into the mechanisms of mRNA turnover. Degradation in the cell is initiated by the addition of U residues to the 3′-end by a poly(U) polymerase [87]. Adding a cordycepin residue (3′-deoxyadenosine) to the 3′-end of synthetic SL-containing mRNA prevents oligouridylation and stabilizes the mRNA. The chapter by Su et al. in this volume presents a detailed protocol for this system.

4 Delivery of Exogenous mRNA to the Cell

A variety of methods have been developed over the years for introducing mRNA into cells. These will be listed here briefly, but several very complete reviews provide more information [9, 10, 88, 89].

4.1 Direct Uptake of Naked mRNA

The first report of protein expression driven by uptake of exogenous synthetic mRNA was by Wolff et al. [6], who injected naked mRNA into mouse skeletal muscle. This mode of delivery is extremely inefficient, but Diken et al. [90] noted that immature DCs are different from most cells in this regard because they take up mRNA by macropinocytosis, being specialized in sampling their

environment by engulfing extracellular fluid. This property made it possible for Kreiter et al. [91] to observe a strong antigen-specific T-cell immunity upon injection of naked antigen-encoding mRNA into mouse lymph nodes. This method can be optimized by co-administration of the DC-activating Fms-like tyrosine kinase 3 (FLT3) ligand as an adjuvant [92]. This protocol is presented in the chapter by Kreiter et al. in the current volume. The antigen-specific response to internodal delivery of naked mRNA is also improved when an mRNA encoding a tumor-associated antigen is injected along with a mixture of mRNAs, termed TriMix, encoding CD40 ligand, constitutively active Toll-like receptor 4, and CD70 [93]. TriMix is utilized in the chapters by Benteyn et al. and Coosemans et al. in the current volume. Rittig et al. [94] performed intradermal injection of naked synthetic mRNA encoding the tumor-associated antigens mucin 1, carcinoembryonic, human epidermal growth factor receptor 2, telomerase, survivin, and melanoma-associated antigen 1 in 30 patients. A clinical benefit was observed in a subset of the patients. Finally, Petsch et al. [95] performed intradermal injection of naked synthetic mRNA encoding the influenza virus HA protein in mice, ferrets, and pigs and observed long-lived and protective immunity to influenza A virus infections.

4.2 Cationic Liposome-Mediated RNA Transfection

Even though uptake of naked mRNA is effective for some applications and cell types, the use of complexing agents generally facilitates uptake and protects the mRNA from nucleases. Malone et al. [96] first used synthetic cationic lipids incorporated into a liposome to transfect mouse NIH 3T3 cells in culture with in vitro-synthesized luciferase mRNA and obtained a linear dose–response of luciferase activity. Conry et al. [97] used this idea to test liposome-protected mRNA as a vector for a tumor vaccine. They synthesized an mRNA encoding human carcinoembryonic antigen, injected mice with it intramuscularly, and found it produced an antigen-specific immune response. Granstein et al. [98] applied the idea of RNA-mediated immunization to the skin. They isolated RNA from S1509a spindle cell tumors, encapsidated it with DOTAP, a commercial liposomal transfection reagent, and used the preparation to pulse CAF1 epidermal cells. When they injected these cells subcutaneously into mice and then challenged the mice with living S1509a cells, they found significantly reduced tumor growth. Kormann et al. [99] used nucleoside-modified mRNA (see below) encased in the cationic lipid preparation Lipofectamine 2000 to express therapeutic proteins. Intramuscular injection of erythropoietin mRNA raised the hematocrit in mice. Application of an aerosol of mRNA encoding surfactant protein B restored the missing protein and permitted survival in a mouse model of a lethal congenital lung disease.

In the current volume, cationic lipids are utilized in the protocols by Boros et al., Bire et al., Devi and Nath, and Lu et al.

4.3 Protamine-Complexed mRNA

RNA can also be protected against degradation by complexing with the polycationic protein protamine. Hoerr et al. [100] synthesized β-galactosidase mRNA and injected the liposome-encapsulated RNA-protamine complex into mice. This led to protein expression, activation of specific cytotoxic T lymphocytes, and production of IgG antibodies against β-galactosidase. Both naked and protected mRNA elicited a specific immune response, but the protected mRNA persisted longer. The protamine-stabilization approach without lipofection reagents was also used in a clinical immunotherapy trial [101]. Protamine-stabilized mRNAs coding for Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3, and survivin in 21 were injected into metastatic melanoma patients. One of seven patients showed a complete response.

4.4 Electroporation and Nucleoporation

Electroporation is another technique that can be used effectively to introduce mRNA into cells ex vivo. Su et al. [102] first introduced mRNA encoding telomerase reverse transcriptase (hTERT) into immature DCs by electroporation and then administered these cells to 20 patients with metastatic prostate cancer. In 19 of them, expansion of hTERT-specific $CD8^+$ T cells was observed in the peripheral blood. Vaccination was further associated with a reduction of prostate-specific antigen velocity and clearance of circulating micrometastases. Since then, introduction of mRNAs into DCs by electroporation has been widely used [9]. It has been possible to achieve large volume electroporation of DCs via continuous flow techniques [103]. Several chapters in this volume use electroporation for various cell types. Idorn et al. use electroporation for tumor infiltrating lymphocytes, Gardner et al. use the technique for fibroblastic and myeloid cells, and Dannull and Nair use it for monocytes. Chapters that use electroporation for DCs are discussed in Subheading 6.2.1.

Nucleoporation is similar to electroporation but uses proprietary nucleofection reagents and introduces nucleic acids into both the cell nucleus and cytosol [104]. It is a gentler procedure than electroporation; cells recover more quickly than for electroporation, and mRNA translation begins as early as measurements can be taken (<10 min) [55, 58, 86]. The chapters in this volume by Su et al. and Koh et al. utilize this technique.

4.5 Other Techniques

Several other techniques have been described for introducing mRNA into cells but are less frequently used than those discussed above. Mandl et al. [105] used a GeneGun to introduce in vitro-synthesized infectious flavivirus RNA, coated onto gold microcarrier particles, into mice, and this induced a protective immunity. Lipid nanoparticles were developed for delivery of siRNAs, miRNAs, and other noncoding RNAs but have been adapted to deliver self-amplifying RNA vaccines [106, 107]. Finally, a self-assembling polyplex nanomicelle composed of a polyethylene

glycol-polyamino acid block copolymer was used to administer luciferase-expressing mRNA with nucleoside modification into the CNS by intrathecal injection into the cisterna magna of mice [108]. Sustained protein expression was observed in the cerebrospinal fluid for one week. Immune responses were suppressed compared with naked mRNA introduction.

5 Exogenous mRNA and the Innate Immunity Response

With the ability to synthesize long, capped mRNAs in vitro, researcher began investigating the expression of specific proteins in cells. This uncovered a new problem: incoming mRNAs are viewed by the cell as viruses and trigger the innate immunity response, leading to accelerated mRNA decay and a shut-off of translation, among other responses [12, 109]. In retrospect, this should have come as no surprise since it had been known since the early 1970s that the dsRNA associated with viral infection shuts down protein synthesis due to activation of PKR, a kinase that phosphorylates initiation factor eIF2α [110], and also induces an endonuclease that degrades mRNA [111]. Ultimately it was discovered that these, as well as other consequences of activating the innate immune response, are due to recognition of both dsRNA [112] and ssRNA [113, 114] by Toll-like receptors.

Shutdown of protein synthesis and degradation of mRNA present a major obstacle to mRNA-based therapeutic approaches. The beginnings of a solution to this problem came from the observation by Karikó et al. [115] that methylated CpG motifs in DNA do not activate the Toll-like receptors. This led them to discover that methylated or otherwise modified ribonucleoside residues such as 5-methyluridine, 5-methylcytidine, N^6-methyladenosine, pseudouridine, and 2-thiouridine also fail to activate the Toll-like receptors. DCs exposed to such modified RNAs expressed significantly less cytokines and activation markers than those treated with unmodified RNA. Hornung et al. [116] then found that RNA containing a 5′-triphosphate is the ligand for retinoic acid-inducible protein I (RIG-I), another receptor that is essential for controlling viral infection. This is particularly relevant for synthetic mRNA that is incompletely capped and therefore retains a 5′-triphosphate (see above). Karikó et al. [117] applied their findings with modified nucleoside residues to the issue of RNA-based therapeutics and found that pseudouridine-containing mRNAs had a higher translational capacity than unmodified mRNAs when tested in mammalian cells and lysates or administered intravenously into mice. Furthermore the substituted mRNAs were more stable. These findings reflect the fact that PKR and endonucleases are not being induced by activation of the innate immunity system. Experiments looking directly at PKR

confirmed that the pseudouridine-for-uridine substitution spares translation by failing to activate PKR [118]. A further improvement in translational efficiency of synthetic mRNA after introduction into cells was obtained by high performance liquid chromatography (HPLC), which removes contaminants like dsRNA in nucleoside-modified mRNA that are responsible for activation of the innate immune response [119]. The purified mRNAs are translated 10- to 1000-fold better in primary cells. The chapter by Boros et al. in this volume utilizes HPLC to purify synthetic mRNA.

Nucleoside-modified mRNAs are now in wide use by those exploring mRNA-based therapeutics. For instance, the production of erythropoietin and surfactant protein B in mice, mentioned in Subheading 4.2, employed mRNA that contained 2-thiouridine, 5-methylcytidine, pseudouridine, and N^6-methyladenosine [99]. In some situations, however, the mRNA is left unmodified intentionally because a vigorous immune response is desired, e.g., in developing vaccines against influenza virus infection [95]. Three protocols in the current volume utilize nucleoside-modified mRNA: Boros et al., Mahiny and Karikó, and Lu et al.

It has long been recognized that, not surprisingly, viruses attempt to thwart host defense mechanism [120]. Recently, Hyde et al. [121] discovered that secondary structures in the 5′-UTRs of many alphaviruses can antagonize host innate immune responses that inhibit translation of nonself mRNAs. This finding potentially could be exploited for therapeutic mRNA. The chapter by Gardner et al. in this volume presents a protocol for synthesizing and transfecting mRNA containing these alphavirus elements as a tool to study the innate immune system.

6 Applications for Synthetic mRNA

Synthetic mRNAs with high translational efficiency, high stability, and low immunogenicity have been used for a number of applications. The following are some broad topics currently being addressed with mRNA-based approaches and some representative studies.

6.1 Protein Replacement to Correct Diseases

Many diseases are caused by the partial or complete absence of a single protein. Alternatively, a protein may be present but have diminished catalytic or regulatory functions. In these cases, delivery of mRNA encoding that protein may be preferable to protein-based therapy. Unlike mRNA-based vaccines, it is disadvantageous in this type of therapy to activate the innate immune system since it shuts down protein synthesis and accelerates mRNA decay. Hence, the use of nucleoside-modified mRNA to suppress this is critical.

Arginine vasopressin was the first protein made with synthetic mRNA in vivo to treat a disease, as noted above [7]. The

Brattleboro rat strain suffers from chronic diabetes insipidus. Injection of a naked, synthetic mRNA encoding vasopressin into the hypothalamus led to selective uptake, retrograde transport, and expression of vasopressin in magnocellular neurons. Reversal of diabetes insipidus was observed for up to 5 days beginning within hours of the injection.

Kormann et al. [99] corrected a mouse model of a lethal congenital lung disease caused by a lack of surfactant protein B, as noted above. They used an aerosol of mRNA encoding surfactant protein B that contained 2-thiouridine and 5-methylcytidine. Twice weekly treatment protected mice from respiratory failure and prolonged their life spans.

Erythropoietin is a practical choice for protein replacement because it is effective at low doses and causes a readily detected increase in hematocrit. Two studies have described injection of nucleoside-modified, HPLC-purified mRNA encoding erythropoietin in mice and primates [99, 122]. The chapter by Mahiny et al. in this volume gives a detailed protocol for measuring hematocrit in mice.

Mays et al. [123] created a disease model of asthma in mice by sensitizing them to ovalbumin. They then delivered a nucleoside-modified mRNA encoding the regulatory T cell transcription factor FOXP3 intratracheally by aerosol. The result was that modified FOXP3 mRNA rebalanced pulmonary T helper cell responses and protected from allergen-induced tissue inflammation, airway hyperresponsiveness, and goblet cell metaplasia. The chapter by Devi and Nath in this volume describes the use of FOXP3-specific cytotoxic T-lymphocytes to treat inflammatory breast cancer cells.

Vascular endothelial growth factor A was expressed from a synthetic mRNA containing pseudouridine and 5-methylcytidine by intramyocardial injection in a mouse model of myocardial infarction [124, 125]. Overexpression of this factor promoted endothelial specification as well as engraftment, proliferation, and survival of human $Isl1^+$ progenitors. This markedly improved heart function and enhanced long-term survival of animals due to mobilization of epicardial progenitor cells and redirection of their differentiation toward cardiovascular cell types.

Finally, expression of CPD-photolyase in human keratinocytes was shown to increase the repair of DNA damage [126]. The chapter by Boros et al. in the current volume presents a protocol for this.

6.2 Vaccines Against Cancer

In 1909, Paul Ehrlich suggested that the immune system may suppress tumor development. Today his prediction is coming true—one of the most exciting and promising applications for synthetic mRNA is immunotherapy for cancer. Because of its great potential, this field has attracted many talented researchers and led to many scholarly publications. A very comprehensive review of the field has been published by Sahin et al. [9].

Furthermore, an entire volume of *Immunological Reviews* containing 17 review articles has been published on this topic, with an introductory chapter by Vonderheide and June [127]. The introductory chapter of the current volume will not attempt to cover this rapidly evolving field but rather will mention only topics that are relevant to the protocols presented here.

6.2.1 Ex Vivo Introduction of Synthetic mRNA into DCs

The idea of using mRNA to program DCs to present tumor antigens for cancer immunotherapy began with the pioneering work of Boczkowski et al. [128], who introduced ovalbumin mRNA into DCs with cationic lipids and then showed that mice vaccinated with these DCs were protected against a challenge with ovalbumin-expressing tumor cells. Seven of the chapters in this volume give protocols relating to introduction of synthetic mRNA into DCs.

Benteyn et al. present methodology to increase the translational efficiency of WT1 mRNA, and Coosemans et al. describe methodology for electroporation of mRNAs encoding WT1, survivin, and TriMix. The Wilms' tumor 1 antigen (WT1) was ranked by the National Cancer Institute as the most relevant for immunotherapeutic targeting [129]. Survivin is an inhibitor of apoptosis that is highly expressed in most cancers and associated with resistance to chemotherapy, tumor recurrence, and shorter patient survival [130]. TriMix is described in Subheading 4.1.

Devi and Nath show how to introduce FOXP3 mRNA into human DCs, generate mature DCs and FOXP3-specific cytotoxic T lymphocytes, and use these T-lymphocytes against inflammatory breast cancer cells. Immunization of mice with DCs transfected with the mRNA for FOXP3, a member of the forkhead winged helix family of transcriptional regulators, stimulates FOXP3-specific cytotoxic T lymphocytes that target the IBC cells of an aggressive subtype of breast cancer [131].

Derdelinckx et al. present a standardized and reproducible method for the manufacturing of GMP-grade mRNA-transfected DCs for clinical use. Selmeczi et al. describe the instrumentation and methods needed for the efficient transfection by electroporation of millions of DCs in one continuous flow process. Borch et al. present a method for performing immune monitoring using peripheral blood mononuclear cells and autologous DCs transfected with tumor antigen-encoding mRNA.

Kreiter et al. [92] discovered that the potency of the immunization can be enhanced if the FLT3 ligand is co-administered with the tumor-associated antigen. They present methods for analysis of FLT3 ligand effects as an adjuvant in combination with antitumor immunization using internodally injected naked mRNA.

6.2.2 Electroporation of T Cells with Synthetic mRNA

Two of the chapters in this volume present protocols in which synthetic mRNA is introduced into T cells by electroporation. Idorn et al. address the problem that only a small fraction of

adoptively transferred T cells reach the tumor site by transfecting tumor infiltrating lymphocytes with mRNA encoding the chemokine receptor CXCR2. Koh et al. seek to redirect T cell specificity towards tumors expressing peptides from hepatitis B virus in hepatocellular carcinoma. They do this by electroporating primary human T lymphocytes with synthetic mRNA encoding a T cell receptors specific for hepatitis B viral antigens expressed on hepatocellular carcinoma cells.

6.3 Vaccines Against Infectious Diseases

The advantages of using synthetic mRNA to immunize against infectious diseases are the same as for cancer—only a transient exposure to antigen-encoding mRNA is needed to prime the immune system, and there is no possibility of integration into the host genome as with DNA. Several successes have been reported for mRNA-based immunotherapy against infectious diseases.

6.3.1 Influenza

The first report of an mRNA-based vaccine was for the influenza A virus [8]. Martinon et al. injected mice subcutaneously with liposome-entrapped synthetic mRNA encoding the nucleoprotein of influenza A virus. The cytotoxic T lymphocytes obtained were indistinguishable from those obtained in vivo with infectious virus in terms of specificity and lysed both peptide-sensitized and virus-infected targets. Petsch et al. [95] made synthetic mRNAs (without modified nucleosides) encoding the neuraminidase and hemagglutinin antigens of influence A virus and administered them by intradermal injection in mice, ferrets, and domestic pigs. They observed B and T cell-dependent protection against multiple influenza antigens. The protective effects were similar to those of a licensed influenza vaccine in pigs. Hekele et al. [107] developed a self-amplifying mRNA encoding the hemagglutinin antigen of influenza A virus. This was complexed with synthetic lipid nanoparticles and used to immunize mice by intramuscular injection. Two weeks after the second immunization, all mice had hemagglutinin inhibition titers that were considered protective.

6.3.2 HIV

An immunotherapy was devised to induce antigen-specific $CD8^+$ and $CD4^+$ T cell responses against HIV-1 proteins [132]. DCs were electroporated with mRNA encoding HIV-1 antigens (Gag, Vpr, Rev, and Nef) without modified nucleosides. The DCs were then injected intradermally into patients every four weeks for four treatments. Full or partial HIV-specific proliferative immune responses occurred in seven of nine subjects. In a second study, patients similarly received four vaccinations with autologous DCs electroporated with mRNA encoding Tat, Rev, and Nef [133]. The patients were then taken off antiretroviral therapy and remained so for 96 weeks. As before, enhanced $CD4^+$ and $CD8^+$ T cell responses specific for the immunogens were observed in most of the patients. In a third study [134], autologous DCs were

electroporated with synthetic mRNA without modified nucleosides encoding Gag or a chimeric Tat-Rev-Nef protein. The DCs were administered to patients four times every four weeks. This caused an increase in the magnitude of the HIV-1-specific IFN-γ response and of T-cell proliferation. The antiviral response to HIV-1 was correlated with the magnitude of the Gag-specific IFN-γ response.

6.4 Engineering the Genome

There are two broad technologies that permit genome editing, which might be considered to be another form of gene therapy: (1) transposases [135] and (2) site-specific nucleases, which are further subdivided into zinc finger nucleases (ZFNs) and truncated transcription activator-like effector nucleases (TALENs) [136]. Both of these technologies can be implemented through introduction of either DNA or synthetic mRNA into cells. Since the transposases and nucleases are only required for a short duration for their action in genome editing, their transient expression from synthetic mRNA serves to reduce off-target consequences as well as unintended genome integration that can occur with DNA vectors. In one example, injecting ZFN-encoding ARCA-capped mRNA without nucleoside modifications into one-cell zebrafish (*Danio rerio*) embryos yielded a high percentage of animals carrying distinct mutations at the ZFN-specified position and exhibiting the expected loss-of-function phenotypes [137]. In another example, injection of either DNA or synthetic mRNA (ARCA-capped, posttranscriptionally polyadenylated) encoding ZFNs into the one-cell rat embryo produced animals carrying 25–100 % disruption at the target locus [138]. This has also been done for mRNA encoding Cas9 [139]. For the transposon termed *Sleeping Beauty*, injection of transposon vectors along with an ARCA-capped mRNA encoding the transposase (without nucleoside modification) into one-cell mouse embryos produced transposition, germ-line transmission, and expression from transposed elements [140]. The chapter by Bire et al. in this volume presents a protocol for producing mRNA encoding the transposase for the *piggyBac* transposon-based system and introducing it into both HeLa and stromal mesenchymal cells.

6.5 Generation of iPSCs

One of the most important biomedical advances of this decade is the ability to convert somatic cells to stem cells by the introduction of DNA encoding a small set of transcription factors [141]. This achievement by Shinya Yamanaka and coworkers, along with much earlier work by John Gurdon on replacing the nucleus of a fertilized *Xenopus* egg with the nucleus of a somatic cell, was recognized by award of the 2012 Nobel Prize in Physiology or Medicine "for the discovery that mature cells can be reprogrammed to become pluripotent". The resulting iPSCs have enormous potential for hematopoietic cell-based therapies and regenerative medicine approaches for the treatment of diabetes, liver disease, neurologic and retinal diseases, muscular dystrophies, and heart disease [142, 143].

Although the initial work of Yamanaka utilized DNA vectors, it was subsequently learned that this could be achieved with synthetic mRNA [13, 144, 145].

Warren et al. [146] prepared synthetic ARCA-capped mRNA with modified nucleosides encoding the four canonical Yamanaka factors, KLF4, c-MYC, OCT4, and SOX2. A poly(A) tract was added with a PCR reaction using a T_{120}-heeled reverse primer. mRNAs were introduced with cationic lipids into murine embryonic fibroblasts, human epidermal keratinocytes, and other cell types. This resulted in reprogramming of cells to pluripotency with efficiencies that greatly surpassed established protocols. The same technology was used to direct the differentiation of these iPSCs into terminally differentiated myogenic cells.

Yakubov et al. [147] prepared synthetic ARCA-capped mRNA encoding the same four transcription factors with a poly(A) tract being transcribed from the plasmid. After five consecutive transfections of human foreskin fibroblasts using a cationic lipid (Lipofectamine 2000), these authors observed formation of iPSC colonies that expressed alkaline phosphatase and several embryonic stem cell markers.

Plews et al. [148] synthesized mRNAs encoding OCT4, SOX2, cMYC, KLF4, and SV40 large T antigen with the AmpliCap-Max™ system, which employs a symmetrical m^7Gpppm^7G cap and provides for poly(A) tract transcription from the plasmid. They electroporated human fibroblast cells with the mixture of mRNAs. After 30 days of culture in human embryonic stem cell medium, they observed small aggregates positive for alkaline phosphatase activity and OCT4 protein.

Subsequent publications have developed and improved the methodology for iPSC generation by synthetic mRNA [149–151]. Lu et al. present a protocol in this volume for introducing synthetic mRNA into human pancreatic progenitor cells as a first step toward their differentiation for transplantation.

7 Concluding Remarks

Paradigm-breaking discoveries and the development of new protocols over the past decade have established synthetic mRNA as a powerful tool in such varied fields as proteins production for disease mitigation, induction of specific immune responses to tumor antigens or infectious agents, editing the genome, and creation of iPSCs for regenerative medicine. Methods have been developed to synthesize mRNAs with increased stability, increased translational efficiency, and decreased immunogenicity for these medical applications as well as for such investigative purposes as photocross-linking to macromolecules, biophysical measurements using fluorescence, and understanding the mechanisms involved in

mRNA synthesis, translation, and degradation. The 20 chapters of this volume presenting protocols cover a range of topics in the broad areas of mRNAs synthesis, introduction into cells, and measurement of the resulting physiological changes. The future looks bright for fulfillment of the initial promise of gene therapy, albeit through RNA-mediated rather than DNA-mediated technologies.

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