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REVIEW

Selection of UTRs in mRNA-Based Gene Therapy and Vaccines

Ilya A. Volkhin[1#], Anastasia Iu. Paremskaia[2,3#], Maria A. Dashian[3], Darya S. Smeshnova[2], Roman E. Pavlov[2], Olga N. Mityaeva[2,4], Pavel Yu. Volchkov[2,5], and Andrei A. Deviatkin[2,6,7,a*]

[1]*Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, 119234 Moscow, Russia*

[2]*Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, 125315 Moscow, Russia*

[3]*Department of Biomedicine, Pirogov Medical University, 117997 Moscow, Russia*

[4]*Moscow Center for Advanced Studies, 123592 Moscow, Russia*

[5]*Center for Personalized Medicine, Loginov Moscow Clinical Scientific Center, 111123 Moscow, Russia*

[6]*Laboratory of Postgenomic Technologies, Izmerov Research Institute of Occupational Health, 105275 Moscow, Russia*

[7]*Federal State Budgetary Institution 'Centre for Strategic Planning and Management of Biomedical Health Risks' of the Federal Medical and Biological Agency, 119121 Moscow, Russia*

[a]*e-mail: devyatkin_aa@academpharm.ru*

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Abstract—The untranslated regions (UTRs) of messenger RNAs (mRNAs) play a crucial role in regulating translational efficiency, stability, and tissue-specific expression. The review describes various applications and challenges of UTR design in the development of gene therapy and mRNA-based therapeutics. UTRs affect critical biological functions, such as mRNA stability, modulation of protein synthesis, and attenuation of immune response. Incorporating tissue-specific microRNA (miRNA)-binding sites into 3′ UTRs might improve precise targeting of transgene expression and minimize off-target effects. Nucleotide modifications (pseudouridine, N1-methyladenosine, and N4-acetylcytidine) in mRNA and UTRs in particular, improve mRNA stability and translational efficiency. At the same time, several challenges remain, such as lack of consensus on UTRs best suited for certain biomedical applications. Current efforts are focused on integrating high-throughput screening, computational modeling, and experimental validation to refine UTR-based therapeutic strategies. The review presents current information on the design of UTRs and their role in therapeutic applications, with special focus on the possibilities and limitations of existing approaches.

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Keywords: gene therapy, mRNA vaccine, UTR, rational design, optimization, RNA secondary structure

INTRODUCTION

The emergence of mRNA-based therapeutics has revolutionized gene therapy and vaccine development by offering promising solutions in the treatment of a broad range of diseases, from genetic disorders to infectious diseases [1, 2]. Currently, there are several approaches to delivering transgenes into human cells for the following protein translation on the delivered mRNA. mRNA vaccines are typically used for a short-term transgene expression, while viral vectors [adeno-associated viruses (AAVs), adenoviruses, lentiviruses, etc.] ensure a long-term expression of the

* To whom correspondence should be addressed.
# These authors contributed equally to this study.

transgene. After virus entry to the cell, transcription of the viral mRNA is regulated by the same processes as transcription of exogenous mRNAs. However, the differences between such mRNAs should not be ignored. For example, modifications of ribonucleotides aimed to reduce the immunogenicity [3] and increase the translational efficiency [4] of artificial RNAs can disrupt their translation [5]. Numerous details in the process of optimizing the structure of viral vectors has been described in several studies [6-9]. However, the issue of UTR design has been rarely discussed [10-12]. At the same time, the studies describing novel approaches to the UTR design [13-16] do not address the challenge of extending such improved UTR design to viral vectors (e.g., AAV vectors). The introduction of best practices in the mRNA design phase of AAV vectors and mRNA vaccines can significantly improve both technologies.

mRNA vaccine encodes a protein (e.g., surface antigen of a pathogen or cancer epitope) that can trigger the human immune response. mRNAs can be encapsulated in lipid nanoparticles, lipid-like materials, polymeric nanoparticles, hybrid systems, and nanoemulsions [1, 17, 18]. These artificial carriers are positively charged and form polyplexes with negatively charged mRNAs, which facilitates their release from the particle into the cell's cytoplasm for further translation [19, 20]. It should be noted that mRNAs are relatively rapidly degraded in the cell. However, their translation yields heterologous proteins that might induce the immune response against the target antigen. The synthesized protein is further processed into smaller peptide epitopes that are presented on the cell surface by the major histocompatibility complexes (MHCs) class I and class II to $CD8^+$ and $CD4^+$ T cells, leading to the development of cellular and humoral immunity, respectively, against the antigen encoded in the mRNA [17, 21]. Due to its high immunogenicity and short lifespan, mRNA is considered to be the optimal vaccine against infectious diseases or cancer [17, 20, 22]. In some cases, mRNA is used as a template for the synthesis of a functional protein that is necessary for the treatment of a specific disease. For example, mRNA-based therapeutics against protein deficiency-related metabolic diseases (propionic acidemia, methylmalonic acidemia, and phenylketonuria) have recently been successfully tested in preclinical studies [23]. Hence, the range of possible applications for mRNA-based drugs can be extended to the development of protein replacement therapies.

Viral vectors (adenoviruses, lentiviruses, retroviruses, AAVs, and others) are typically used for the long-term transgene production a with a direct therapeutic effect [24] which is achieved by maintaining the viral genome through its constant transcription. This process results in the intracellular synthesis of mRNA utilized for the production of a desired protein. The precise dosage of the virus is determined, in particular, by the efficiency of protein synthesis, as higher efficiency of transcription and translation allows to use a lower virus dose, which is safer and more cost-effective [25]. Due to the high efficacy, AAV vectors have been used much more commonly than other viral vectors [26, 27]. For this reason, this review focused mostly on AAV-based gene therapeutics. Recombinant AAV (rAAV) vectors, which lack the viral *rep* and *cap* genes, carry an expression cassette consisting of the transcription promoter, single-stranded DNA coding for the transgene and flanked by inverted terminal repeats, and polyadenylation signal (polyA) within an icosahedral capsid that can allow tissue-specific targeting. After endocytosis, viral single-stranded DNA of the forms episomes in the nucleus and then converted into the double-stranded DNA transcribed by the cellular machinery. The mRNA is exported to the cytosol for the translation of the therapeutic transgene [27, 28]. The lifespan of the transgene introduced to the cell by this approach is limited only by the lifespan of the cell itself [21 29]. For instance, in tissues with a low cell turnover (e.g., muscles), expression of the introduced transgene (factor IX) has been detected for at least 10 years [30]. Consequently, the use of AAV vectors is currently the preferred method for producing intracellular functional proteins to compensate for defective endogenous proteins in the cytoplasm. The application of AAVs as delivery vectors for the gene replacement therapy has been supported by clinical trials and FDA approvals for their use in the treatment of ocular, neurological, metabolic, and hematological disorders [27, 31-35].

The above therapies rely directly (mRNA vaccines) or indirectly (AAV vectors) on mRNA as a template for protein synthesis. It should be noted that mRNAs transcribed on the AAV DNA in the nucleus and then exported to the cytoplasm are structurally similar to heterologous mRNAs that enter the cell from the external environment (Fig. 1). Both types of mRNAs have similar structural domains that contain all essential elements required for their translation, namely, the 5′ cap, 5′ untranslated region (UTR), open reading frame (ORF), 3′ UTR, and poly(A) tail [1, 20, 36]. At the same time, the mechanism for exogenous RNA recognition by the cellular machinery can be different from that for endogenous RNAs. RNA synthesized *in vitro* may not have the nucleotide composition and secondary structure of natural RNAs. As a result, external RNAs can trigger the mechanisms of intracellular immunity after recognition by the corresponding RNA-binding proteins [37]. Moreover, the process of RNA synthesis *in vitro* is not precise and can lead to the formation of double-stranded RNAs [38] and truncated RNAs (aborted transcription products) [39].

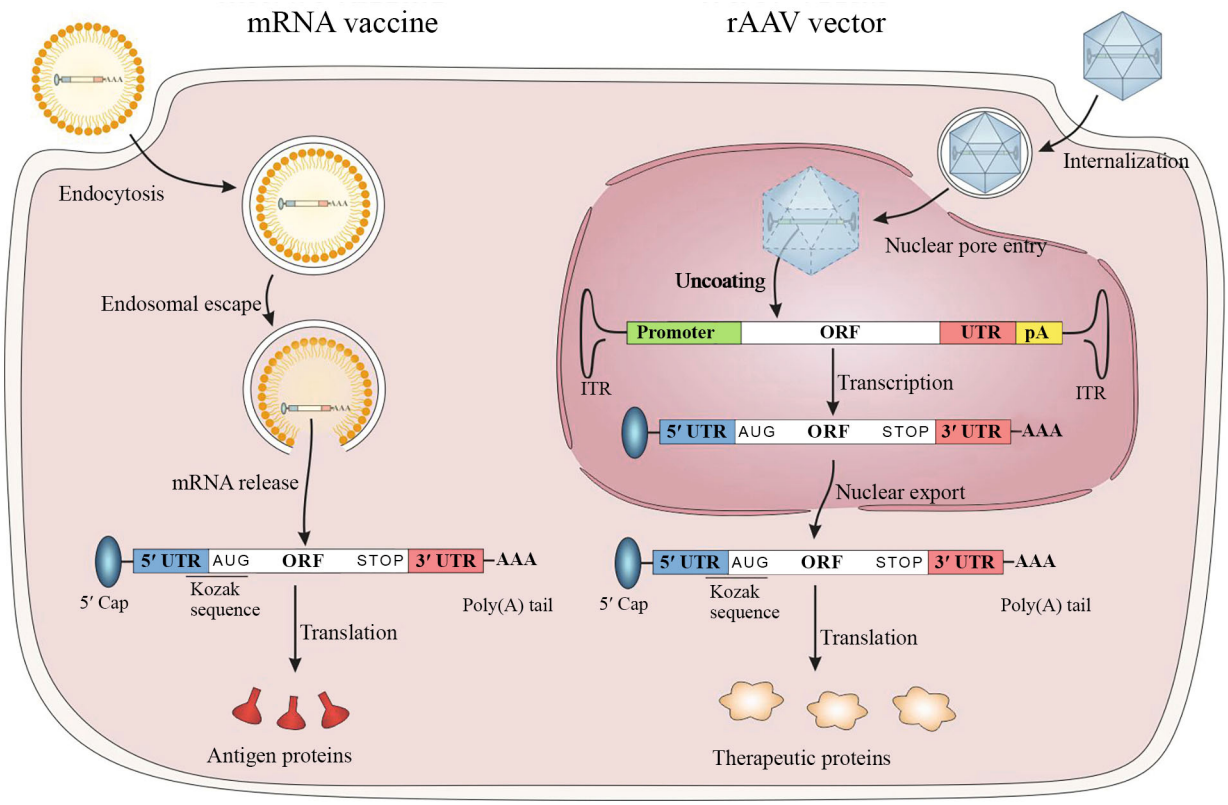

Fig. 1. mRNA-based therapeutics: mRNA vaccine (left) and rAAV vector-based drug (right). The mRNA vaccine contains ready-for-translation mRNA encapsulated in a liposomal particle. Upon release from the liposome inside the cell, the mRNA is translated by the cell ribosomes, and the synthesized polypeptide serves as an antigen that stimulates the host's immune response or as a therapeutic protein compensating for a defective cellular protein. The rAAV vector consists of a single-stranded DNA carrying a transgene inside a viral capsid. After the vector enters the cell via endocytosis, the rAAV genome is transcribed in the nucleus. The resulting mRNA is exported to the cytoplasm, where it is translated into the therapeutic protein.

Numerous efforts have been made to minimize these factors (see review [37]).

After mRNA entry to the cytoplasm, its 3′ poly(A) tail and 5′ cap interact with the translation initiation factors (eIFs), leading to the mRNA circularization and recruitment of the preinitiation complex (PIC) formed by the 40S small ribosomal subunit, eIFs, initiating methionyl-tRNA, and GTP. The 40S small ribosomal subunit scans the 5′ UTR for the start codon in the Kozak sequence. After the start codon is found, the eIFs are released and the 60S ribosomal subunit is recruited to form the 80S ribosome capable of protein synthesis. The elongation of the amino acid chain continues until the stop codon is reached. Finally, the nascent protein gains functionality through post-translational modifications [1, 17, 40]. It should be noted that translation requires the presence of all mRNA structural domains (5′ cap, 5′ UTR, Kozak sequence, ORF, and 3′ UTR). Therefore, altering any mRNA domain can directly affect the stability, immunogenicity, and translation of mRNA therapeutics [20, 41]. Currently, there are a variety of approaches for optimizing the 5′ cap, nucleotide context of the start codon, and poly(A) tail [42-58]. For example, the length of the poly(A) tail can influence the efficiency of mRNA translation and mRNA degradation rate [45, 50-52]. Interestingly, the effect of the poly(A) tail on translation depends on the length of the 3′ UTR [59, 60]. The stability of mRNA can also be affected by the chemical modifications of the poly(A) tail [61, 62]. However, this review primarily focuses on the strategies for optimizing the UTRs.

Despite a significant progress in the development of mRNA-based therapies and vaccines, there is a lack of consensus on the optimal UTRs to be used in specific biomedical applications. UTRs, which flank the coding sequence in mRNA, are crucial for regulating the translational efficiency and stability of mRNA. Commonly, the UTRs selected for therapeutic applications (e.g., UTRs of human β-globin mRNA) provide a high translational efficiency and mRNA stability. Consequently, the optimization of UTRs has emerged as an area of focus in the development of mRNA-based therapeutics [63, 64].

In this review, we analyzed in detail the UTRs that have shown promise in preclinical and clinical studies in order to identify the strategies for selection

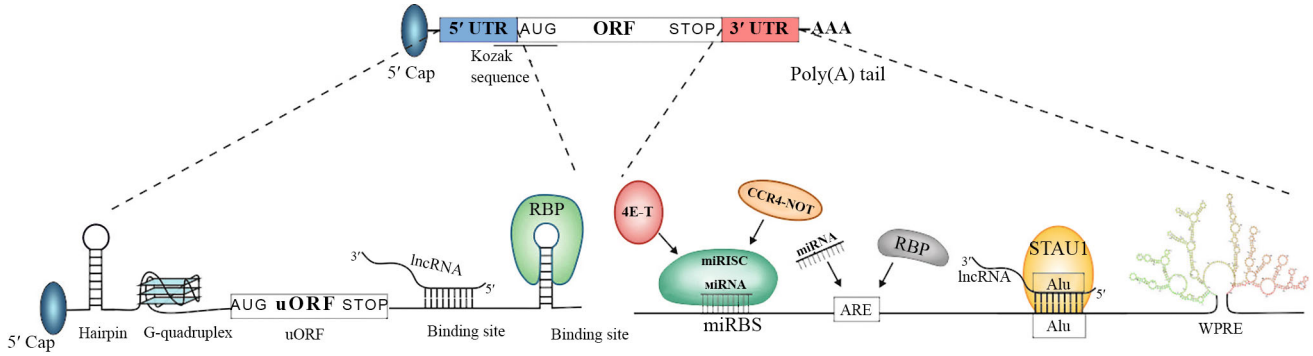

Fig. 2. Regulatory structures in the 5′ UTR and 3′ UTR that influence translational efficiency and mRNA stability. Abbreviations: uORF, upstream open reading frame; lncRNA, long non-coding RNA; miRBS, miRNA-binding site; miRISC, miRNA-induced silencing complex; 4E-T, eukaryotic translation initiation factor 4E transporter; CCR4-NOT, carbon catabolite repression – negative on TATA-less; STAU1, staufen double-stranded RNA binding protein 1; ARE, AU-rich element; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; RBPs, RNA-binding proteins.

of most effective UTRs for therapeutic applications and to guide researchers in choosing the optimal UTRs for their specific needs.

THE EFFECT OF UTRs ON THE PROTEIN YIELD

The translational efficiency (translation rate) is often defined as the number of protein molecules produced per mRNA molecule per unit of time [65]. The mRNA degradation rate is defined as the number of mRNA molecules degraded per unit of time, reflecting the speed rate at which mRNA is eliminated from the cell and can be influenced by multiple factors, such as mRNA sequence elements, RNA-binding proteins (RBPs), microRNAs (miRNAs), and various decay mechanisms.

The goal behind the generation of synthetic mRNAs is usually to achieve a higher protein yield, which depends on both mRNA translation efficiency and degradation rate. The half-live time differs for different mRNAs, with a median value of approximately 7 h [66]. There is an inherent trade-off between the translation rate and mRNA stability [67]. Although high ribosome loading increases the translation rate, it can also make mRNA more susceptible to hydrolysis. The reasons for this phenomenon remain unknown, but might involve ribosome crowding and consequent mRNA degradation. In fact, stable mRNAs, even if they have a lower translational efficiency, can ultimately produce more protein over time due to a longer lifespan [67]. Therefore, balancing translational efficiency and mRNA stability is critical, and the choice of UTRs plays a pivotal role in modulating these factors.

The efficiency of translation is influenced by the stability and position of the secondary structure elements (e.g., hairpins) within the 5′ UTR (Fig. 2). The rate-limiting step of translation initiation is the binding of the 43S PIC to mRNA [68]. Since stable secondary structures in the 5′ UTR can prevent this process, it is generally assumed that they suppress translation. As the stability of the hairpin increases, the translational efficiency typically decreases. For example, an increase in the hairpin stability from –25 to –35 kcal/mol can almost completely halt the translation [68]. It has also been shown that hairpins located close to the 5′ cap strongly repress translation [69], likely by interfering with the mRNA recognition or PIC binding. Furthermore, the guanine–cytosine (GC) content of the hairpin influences the translational efficiency independently of the hairpin thermal stability or position [68]. The simplest explanation is that the ribosome does not melt the entire hairpin at once but unwinds it gradually. The GC pairs are more difficult to break apart than the AU pairs because of their higher stability due to an additional hydrogen bond and, therefore, can slow down or delay the progress of the ribosome. The hairpins with the same stability but a higher GC content have a stronger inhibitory effect on the translational efficiency. Even though secondary structures typically reduce the efficiency of translation, some 5′ UTRs with stable secondary structure elements can support efficient initiation of translation, for example, the 5′ UTR of dengue virus (DEN2) [67], presumably, due to the promotion of efficient cap-independent translation initiation by the secondary structure [70]. Another probable explanation is that the 5′ UTR of DEN2 interacts with the cellular protein La, which acts as an RNA helicase and thus facilitates translation [71]. In summary, it is usually better to avoid formation of stable secondary structures in the 5′ UTRs of artificial mRNAs, especially near the start codon and the cap.

Generally, stable elements of secondary structures increase RNA stability in solution by protecting it from hydrolysis [72]. mRNA molecules in the intracellular environment are generally less structured

than *in vitro*, due to the interactions with RBPs, RNA helicases, and ribosomes [73], which highlights the importance of cellular context when evaluating the structural features of RNA.

G-quadruplexes (RG4s) are a specific type of transient four-stranded structures in RNA that can impact translation [74]. The exact mechanism of their influence remains unknown, but it has been hypothesized that RG4s inhibit translation by preventing the binding of the 43S PIC to mRNA or by slowing down the scanning [73]. However, there is also evidence of a positive influence of RG4s on translation (e.g., in the case of human *clAP1* mRNA) [75]. Therefore, the impact of RG4s on translation appears to be multidirectional, and its underlying mechanisms still have to be elucidated in future studies.

Upstream open reading frames (uORFs) are short reading frames located upstream of the major coding sequence; they are common regulatory sequences in human 5′ UTRs [76-80]. Although ribosomes can reinitiate translation downstream of uORFs, these elements are thought to have a strong inhibitory effect on translation [81, 82]. The simplest mechanism of such inhibition is recognition of the uORF instead of the canonical ORF, which decreases the probability of translation initiation for the canonical protein. Another mechanism is ribosome stalling of by a peptide encoded by such uORF, as was shown for human cytomegalovirus UL4 mRNA [83]. Beside decrease the translational efficiency, uORFs cause mRNA degradation via the nonsense-mediated mRNA decay mechanism [84, 85] initiated by the presence of a premature stop codon [86]. It was demonstrated that an uORF within the 5′ UTR of mRNA can activate the process, ultimately resulting in mRNA degradation [86, 87]. At the same time some UTRs contain signals that protects mRNA from degradation despite the presence of uORFs, as it was shown for yeast mRNAs [88]. The upstream start codons (uAUGs) that do not form complete uORFs within the 5′ UTR (i.e., lack the in-frame stop codon before the main AUG) have an even stronger inhibitory effect on translation than complete uORFs. The reason for this might be the inability of ribosomes to reinitiate translation from the main ORF downstream of such uAUGs [76]. Since uORFs and uAUGs usually have a strong negative impact on translation, they should be absent in artificial mRNAs.

The presence of binding sites for proteins and long non-coding RNAs (lncRNAs) in the UTRs can also affect translation due to the interaction of these lncRNAs and RBPs with the translation machinery [73, 89]. For example, the antisense *Uchl1* lncRNA promotes the synthesis of mouse ubiquitin carboxy-terminal hydrolase L1 (UCHL1) due to the enhanced ribosome binding [90]. The RBP-binding sites can either enhance or inhibit translation. An example of the RBP-mediated translational regulation is the role of iron regulatory proteins 1 and 2 (IRP1 and 2). These proteins can bind to specific regions (known as iron response elements) in the 5′ UTRs of mRNAs coding for iron metabolism proteins, thus inhibiting their translation [91]. Another example is regulation of the *msl-2* mRNA translation by the sex-lethal (SXL) protein in *Drosophila*, which was found to bind both the 5′ and 3′ UTRs of *msl-2* mRNA and to inhibit its translation. However, the mechanisms of inhibition differ for the 5′ and 3′ UTRs. In the 3′ UTR, SXL recruits the UNR (upstream of N-Ras) repressor protein and prevents the formation of PIC. The binding of SXL to the 5′ UTR requires the presence of the binding site and the uORF and promotes the recognition of uAUG, thus inhibiting translation of the main ORF [92]. An example of the positive effect of RBPs is activation of the cap-independent translation by the YB-1 protein [93]. Some GC-enriched clusters present in particular human mRNAs can recruit YB-1 and promote translation. In summary, the interaction of UTRs with lncRNAs and RBPs may also play a role in the translational regulation. Therefore, potential interactions of the binding motifs present in the UTRs should be taken into account when selecting the UTRs for the use in mRNA vaccines and mRNA-based agents.

In contrast to the 5′ UTR, the 3′ UTR are believed to have a lesser influence on the ribosome loading and, therefore, translation rate [67]. However, the 3′ UTRs may contain some regulatory sequences capable of interacting with RBPs or microRNAs (miRNAs) and influencing the efficiency of mRNA translation and lifespan [66, 94]. Their effect could be explained by several mechanisms. For example, the CCR4-NOT exonuclease can inhibit translation and cause mRNA decay. It is recruited by RBPs and miRNAs interacting with specific sequence motifs in the 3′ UTR. Hence, a 3′ UTR lacking these sites will not be degraded by CCR4-NOT. One of the mechanisms of CCR4-NOT action is physical displacement of poly(A)-binding proteins (PABPs) and prevention of translation initiation [95]. Another mechanism is the recruitment of special inhibitor proteins, such as eukaryotic translation initiation factor 4E transporter (4E-T), which interacts with the translation initiation factors and inhibits translation initiation [96]. CCR4-NOT has also been demonstrated to induce mRNA decapping and deadenylation, leading to mRNA degradation.

Another mechanism through which 3′ UTRs exert their influence on mRNA decay is the presence of adenylate/uridylate (AU)-rich elements (AREs) in the 3′ UTRs. These sequences are considered to be mRNA decay signals [94]. It has been demonstrated that many RBPs, including tristetraprolin and butyrate response factors 1 and 2, bind to AREs, resulting in mRNA destabilization and degradation [97]. However,

not all AU-rich elements cause translational repression. Thus, the human antigen R protein (HuR) binds AU-rich sequences in the 3′ UTRs, thereby increasing the stability of the corresponding mRNAs by protecting them from miRNA binding through steric hindrances [98]. The precise mechanism of ARE influence on the mRNA stability depends on the stoichiometry between AREs and RBPs, as well as the 3′ UTR structure and arrangement of regulatory elements. Furthermore, mRNA cleavage can be facilitated by lncRNAs. For instance, 3′ UTRs of some mRNAs contain Alu elements that can pair with certain lncRNAs, resulting in the formation of double-stranded RNAs that undergo degradation via the STAU1 (staufen double-stranded RNA binding protein 1)-mediated mechanism [99].

Other molecules that can promote mRNA degradation are miRNAs which guide the miRNA-induced silencing complex (miRISC) composed of the miRNA and Argonaute protein [100]. miRISC binds to specialized sites on mRNAs, known as miRNA-binding sites (miRBSs), and recruits effector protein complexes, such as PAN2-PAN3 and CCR4-NOT, that inhibit translation and cause mRNA degradation. Therefore, to ensure a greater mRNA stability, it is important to avoid the degradation signals (AREs, Alu elements, and miRBSs) in the 3′ UTRs. However, the effect of such signals depends on the intricate cellular signaling network and can be almost unpredictable. Interestingly, Leppek et al. [67] have shown that the 5′ UTRs have a greater effect on the mRNA stability than the 3′ UTR. This is surprising because there is a plethora of mechanisms by which the 3′ UTR can influence the mRNA lifespan. To the best of our knowledge, the reason for this phenomenon is unknown. The 3′ UTRs can either inhibit or promote translation. For instance, the 3′ UTRs of mRNAs coding for some nuclear-encoded mitochondrial proteins (e.g., ATPase subunits) promote translation due to the interactions with the translation machinery [60, 101]. Another example is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which is frequently used in gene therapy to increase protein production. While its main function is to promote the nuclear export of mRNAs [102], Loeb et al. [103] suggested that WPRE can also activate translation via an unknown mechanism.

UTR DESIGN FOR TISSUE-SPECIFIC EXPRESSION

Approximately half of human genes produce multiple mRNA isoforms that can vary in their 3′ UTR sequences [104]. Most mRNAs with a single isoform are transcribed in a tissue-restricted manner, whereas ubiquitously expressed genes are typically transcribed into multiple mRNA isoforms, depending on the tissue context. This tissue-dependent diversity of 3′ UTRs can be potentially exploited to develop tissue-specific gene therapies. Limiting expression of transgenes in non-target tissues is critical for preventing the toxic effects of protein overexpression and immunotoxicity. Theoretically, this can be achieved by incorporating tissue-specific miRBSs into 3′ UTRs in order to restrict transgene expression in the cells enriched in the corresponding miRNA (Fig. 3). For example, it was reported that adding the binding sites for miR-122 and miR-1 to rAAV9 promoted protein synthesis in the CNS while reducing its expression in the liver, heart, and skeletal muscles. The incorporation of miR-204 and miR-124 targeted transgene expression to the photoreceptors and retinal pigment epithelium, respectively [27, 105-108].

miRNA targeting is an effective way to restrict the transgene expression to specific tissues based on different miRNA profiles of different cell lineages. Ludwig et al. [109] created a comprehensive human miRNA tissue atlas by determining the abundance of 1997 miRNAs in 61 tissue biopsies of different organs. One thousand three hundred sixty-four miRNAs were discovered in at least one tissue, 143 were present in each tissue. The majority of miRNAs displayed intermediate tissue specificity, with some expressed predominantly in specific tissues, such as the brain [109]. Recent studies have shown that the tissue tropism of certain viruses could be eliminated by the incorporation of miRNA target sequences into their genomes to prevent viral RNA translation in the tissues expressing tissue-specific miRNAs. Vesicular stomatitis virus is considered as a promising recombinant vaccine platform and oncolytic agent. However, it has not yet been tested in humans, as it was found to cause encephalomyelitis in rodents and primates by almost any route of administration. Kelly et al. [110] attempted to eliminate this effect by creating an attenuated viral strain by inserting neuron-specific miRBSs into the virus 3′ UTR. The new viral strain retained its original oncolytic properties, but lost the neurotropic effect, which allows its use in clinical trials. This approach has been successfully applied to the poliovirus [111] and mosquito-borne Japanese encephalitis virus [112]. The modified strains retained their ability to replicate in non-neuronal tissues, but their neurotropism decreased significantly, leading to the development of safer and more efficient vaccines.

5′ UTRs may display tissue specificity due to the alternative splicing and formation of multiple variants of the first exon encoding different 5′ UTRs [113, 114], the use of multiple promoters [115, 116], or a combination of these mechanisms. While the resulting mRNAs have identical coding regions, the presence of different 5′ UTRs affects the translational efficiency, leading to the tissue-specific expression of the same gene. However, this regulatory mechanism

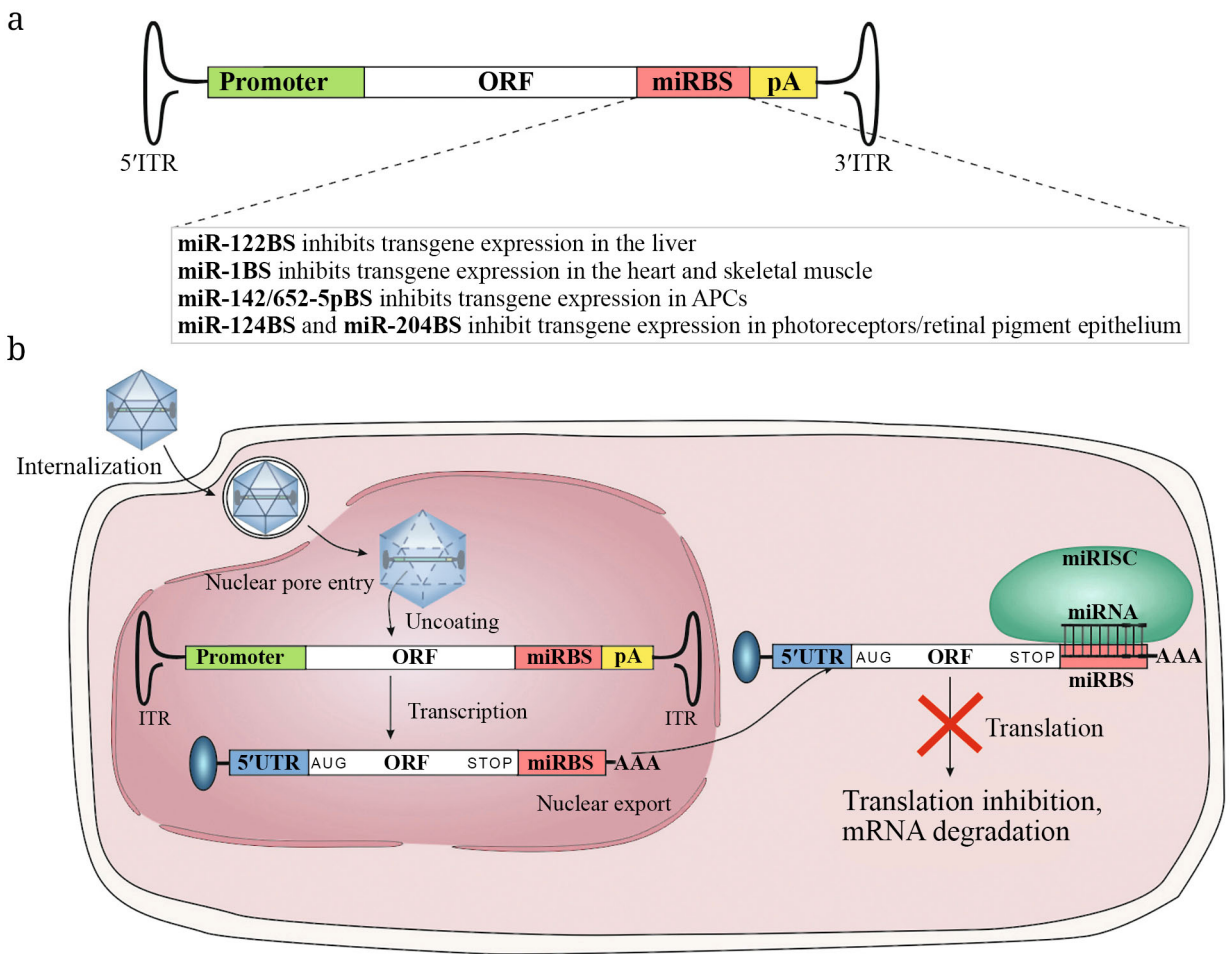

Fig. 3. Principles of tissue-specific 3′ UTR design. Although this approach has been successfully demonstrated for AAV vectors, it can also be applied to mRNA therapies. a) The AAV vector expression cassette contains the binding sites for tissue-specific miRNAs in the 3′ UTR. Incorporation of miRBSs for miRNAs present in the non-target tissues or cells allows to reduce off-target transgene expression. b) The AAV vector is internalized into the cell and enters the nucleus, where the transgene mRNA is transcribed. After mRNA transcription and nuclear export, tissue-specific miRNA in the content of miRISC binds to miRBSs in the synthesized mRNA, resulting in silencing due to the translational repression and/or mRNA degradation in the off-target cells.

is not directly applicable to the development of mRNA vaccines or gene therapies, as the 5′ UTRs in such constructs are synthetic and are not the subjects to endogenous transcriptional regulation. Once an mRNA enters the cell, transcription no longer occurs and translation depends on the cellular environment and properties of mRNA itself. Although this mechanism is informative from a biological point of view, it cannot be used for tissue targeting in the development of mRNA-based therapeutics.

The major challenge in protein replacement therapy is the immune response triggered by heterologous proteins, which can undermine the efficacy of therapy. For example, AAV vectors can deliver transgenes not only to target cells, but also to antigen-presenting cells (APCs). APCs process foreign proteins and initiate the immune response. A promising strategy to mitigate this issue involves incorporation of binding sites for APC-specific miRNAs, such as miR-142 and miR-652, into the rAAV expression cassette to redirect the transgene expression away from the APCs. The miRBSs engineered for the transgene can interact with cellular miRNAs, leading to the transcript degradation. In APCs, such constructs can prevent the presentation of transgene peptides to T cells, thus reducing immunity against the transgene protein [117-119]. PARADE (Prediction and RAtional DEsign of mRNA UTRs) is a recently developed promising tool [120] for designing UTRs regions with a tailored cell type-specific activity. To develop this generative artificial intelligence framework, the regulatory activity of 60,000 5′ and 3′ UTRs was measured in six cell types. The PARADE tool was validated by testing 15,800 *de novo* designed sequences in six cell types. Many sequences showed superior specificity and activity compared to existing RNA therapeutics. The PARADE tool can potentially

facilitate the design of tissue-specific mRNAs for various therapies by mitigating the negative effects of protein expression in non-target organs.

THE IMPACT OF MODIFIED NUCLEOTIDES IN THE UTRs

The dynamic regulation of post-transcriptional modifications (PTMs) is mediated by a complex network of 'writer', 'reader', and 'eraser' proteins [121]. PTMs play a critical role in RNA preprocessing in the nucleus, including alternative splicing and nuclear export. In the cytoplasm, PTMs modulate important aspects of the transcript life cycle, such as intracellular localization, translational regulation, stability, and degradation [122].

Current studies on the epigenetic regulation of RNAs by PTMs primarily focus on their impact in cancer therapy [123] and involvement in the development of complex neurological, cardiovascular, and metabolic diseases [122, 124]. However, PTMs may also hold significance for the future development of mRNA-based therapeutics. Thus, Pfizer and Moderna used PTMs in the development of the COVID-19 mRNA vaccine [125]. The mRNA component of the vaccine was composed of the 5′ UTR, the coding sequence for the spike protein with two consecutive stop codons, and the 3′ UTR in which all uridines have been replaced by N1-methylpseudouridine.

The distribution of nucleotide modifications across different regions of mRNA varies significantly. For example, pseudouridines are more prevalent in the 3′ UTRs and coding regions than in the 5′ UTRs [126]. *In vivo* isomerization of uridine to pseudouridine is carried out by pseudouridine synthases (PUSs). PUSs catalyze the formation of pseudouridine by breaking the N1–C1′ glycosidic bond and forming a new C5–C1′ bond, with the N1 atom becoming an additional hydrogen bond donor. The resulting conformational changes contribute to the stabilization of the polynucleotide chain [127]. mRNAs containing pseudouridine and 1-methylpseudouridine have a reduced ability to activate the innate immune system because they are less likely to be detected by receptors, such as the Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene 1 (RIG-I) [3, 128, 129]. According to predictions from the convolutional neural network trained on unmodified sequences, the incorporation of pseudouridine and 1-methylpseudouridine into the 5′ UTRs correlates positively with the ribosomal loading due to the increase in the minimum free energy and alterations in the RNA secondary structure [130]. However, N1-methylpseudouridine induces ribosome pausing and leads to a +1 frameshift [5]. Pseudouridylation is associated with the enhanced mRNA translational efficiency. Thus, it was found that PUS7 promotes the translation of the tumor suppressor ALKBH3 in gastric cancer [131]. The translational efficiency of modified transcripts depends on the extent of modification: the transcripts containing over 50% N1-methylpseudouridine substitutions yielded less protein than those with lesser percentage of modification [129]. As demonstrated in a recent study, N1-methylpseudouridine in a context-dependent manner affected the decoding of the mRNA sequence by altering the strength and pattern of the mRNA–tRNA interactions in the ribosome A site [132]. 5-Methoxyuridine (5moU), an alternative modification of uridine, increased the stability, reduced immunogenicity, and enhanced protein yield for *in vitro* transcribed (IVT) mRNA [133].

When located in different transcript regions, the same modification can perform different biological functions. For example, the enrichment of the start codon, stop codon, and 5′ UTR (particularly at the critical cap+1 and cap+2 positions) with N^1-methyladenosine (m^1A) enhances the translational efficiency, whereas modification of the coding sequence and the 3′ UTR reduced the efficiency of translation [134-136]. It was also found that m1A in the 3′ UTR impacts the interaction between mRNA and miRNAs; however, the underlying mechanisms of this influence are yet poorly understood [137]. The m^1A modification enhances the effect of N^6-methyladenosine (m^6A) by inducing the RNA degradation pathway [138]. The m1A sites attract HRSP12 (heat-responsive protein 12), which facilitates the binding of the m^6A reader protein YTHDF2 (YTH domain family 2) to m^6A sites on the same RNA molecule. The HRSP12–YTHDF2 complex recruits the P/MRP endoribonuclease that cleaves mRNA thus suppressing translation [139, 140]. The m^6A modification promotes destabilization of the RNA secondary structure [141]. Early studies have indicated that its presence in the 5′ UTR near the start codon facilitates the cap-independent translation. However, recent findings show that the thermodynamic changes resulting from a single modification are insufficient to stabilize the initiation complex and do not affect the translational efficiency [142]. The effect of the m^6A modification on the translation initiation may be mediated by the reader protein YTHDF1. YTHDF1 binds to m^6A sites near the 3′ UTR proximal region and, through loop formation, interacts with eIF3. YTHDF3 interacts with YTHDF1 to enhance its translation-promoting effect [143].

N4-acetylcytidine (ac4C) had been originally identified in tRNA and rRNA; its presence mRNA was confirmed later. This PTM plays a critical role in regulating the transcript stability and gene expression. Overexpression of NAT10, an enzyme catalyzing ac4C modification, was found to promote proliferation of malignant cell in many cancer types [144]. It has been

repeatedly demonstrated that ac4C, which predominates in the 3′ UTR and surrounds the stop codon, can stabilize mRNA [145, 146]. The presence of ac4C in the coding sequence is associated with the upregulated expression of the corresponding mRNA [147]. Also, the presence of ac4C in the 5′ UTR influences translation initiation by suppressing the usage of strong start codons and facilitating alternative initiation at weaker sites [148].

NSUN proteins are a family of RNA methyltransferases that catalyze the formation of 5-methylcytosine (m^5C), thus playing a critical role in RNA metabolism. m^5C is found throughout the transcripts, with a higher concentration in the 5′ UTRs. Interestingly, m^5C-containing mRNAs demonstrate a reduced translational efficiency, despite longer half-life [149]. The content of N^7-methylguanosine (m^7G) in the coding sequence and 3′ UTR increases after heat shock and oxidative stress, while the content in the 5′ UTR decreases, which contributes to the increased translational efficiency of such mRNAs [150]. m^7G recruits initiation factors and protects RNAs from 5′ exonucleases [151].

Therefore, incorporating modified bases in the UTRs and coding sequencing, as well as taking into account the functional properties of modified nucleotides, including their ability to trigger or attenuate the immune response, regulate translation rate and efficiency, and increase mRNA stability, can improve the development of therapeutic mRNAs. However, it is important to point out that the existing data are limited, and further research is needed for a more comprehensive understanding of the mechanisms underlying the effect of modified bases.

UTRs IN RESEARCH AND CLINICAL TRIALS

The development of gene therapeutics and mRNA vaccines can be compared to the construction of a building from different ‘building blocks’, for example, 3′ and 5′ UTRs, which can be tailored and combined with the coding sequences to optimize the resulting product.

One strategy to increase the expression efficiency and stability of IVT mRNAs is the use of UTRs from highly translated, constitutively expressed, and stable cellular endogenous genes [67]. To achieve high translational efficiency, many mRNA therapies utilize the well-studied 3′ and 5′ UTRs of the human α- and β-globin genes [68, 152-159] and rabbit β-globin genes [160], as they improve the translation efficiency and increase the stability of heterologous mRNAs [16, 161-163].

It is believed that the 5′ UTR of β-globin mRNA ensures efficient cap-dependent translation due to the lack of stable secondary structures or translation-suppressing elements [68]. The 3′ UTRs of the α- and β-globin mRNAs provide a high stability of mRNA due to association with RBPs [164-168]. For this reason, a combination of 5′ and 3′ UTRs from the globin mRNAs is widely used in the studies of translation and stability of IVT mRNA [67, 157], creation of vaccines for immunotherapy [152, 153, 161, 169], and cell reprogramming into induced pluripotent stem cells (iPSCs) [154]. Two 3′ UTRs the from human β-globin mRNA cloned head to tail were found to be superior to a single 3′ UTR in improving mRNA translation and stability, inducing immune responses in mice. The efficiency of such constructs is currently tested in ongoing clinical trials (ClinicalTrials.gov: NCT01684241, NCT02035956, NCT02316457, NCT02410733, and NCT03418480) [170].

In addition to the widely used α- and β-globin UTRs, UTRs from other genes can also be incorporated into mRNAs for mRNA-based therapeutics. Some natural UTRs have been shown to improve the efficiency of the corresponding products. For example, the UTRs of the *TSMB10*, *TMSB4X* [171]; *HBB*, *H4C2*, *Rabb*, *TPL* [163], *mtRNR1*, *AES* [172]; *CYBA* [173], *Rps27a* [44], *C3*, *CYP2E1*, and *APOA2* [63] genes, viral UTRs (e.g., from dengue virus) [174], TEV and TMV 5′ UTRs [67, 175], and many others have been found to improve the efficacy of therapeutics and can be potentially used in developing the UTR design strategies aimed to improve the mRNA translational efficiency. The IRES (internal ribosome entry site)-containing 5′ UTR from the encephalomyocarditis virus enhanced transcription of uncapped mRNAs and increased transgene expression in human and murine cultured dendritic cells (DCs). Immunization of mice with DCs transfected with the IRES-containing mRNA induced high levels of antigen-specific T cells, promoted elimination of antigen-bearing cells, and protected immunized mice from pulmonary metastasis of melanoma cells injected intravenously, suggesting a potential use of IRES-containing 5′ UTRs in the antitumor immunotherapy [176]. The IRES was also included in the IVT mRNA used in the reprogramming of human fibroblasts into pluripotent stem cells [177]. Some regulatory motifs, such as translation initiator of short 5′ UTRs (TISU) [178], may also enhance mRNA translation. However, all publications indicated that the observed effects were cell type-specific [163]. The list of studied 3′ and 5′ UTRs and their effects on the translational efficiency and mRNA stability are summarized in Tables 1 and 2.

Due to the emergence of new computational methods and machine learning, artificially generated UTRs with specified properties can be currently developed to provide an increased mRNA stability and efficient protein production compared to natural endogenous UTRs. Thus, the creation of artificial 5′ UTRs ensuring high translational efficiency was described in [185].

Table 1. Studied 5′ UTRs and their impact on the translational efficiency and mRNA stability

Selected 5′ UTR	Advantages	5′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
α-globin	α-globin UTR demonstrated higher translational efficiency in living cells, whereas lower values were observed in *in vitro* lysates	β-globin	Cos-7 cells	origin: human α-globin mRNA length: 34 nucleotides (nt) [179]	[68, 180]
	α-globin UTR enhanced mRNA stability and protein translation	*CYBA* *ACE2*	A549 and HepG2 cells		[164]
Human *Hsp70*	UTR increased the efficiency of mRNA translation in cultured cells and enhanced antibody response *in vivo*	*Grp 78* *VEGF*	HepG2 cells Balb/c mice	origin: human *Hsp70* mRNA length: 213 nt	[181]
HBB *HSPA1A* *Rabb* *H4C2*	UTRs increased the translational efficiency and protein level	5′ UTR of mRNA-1273 (Moderna) 5′ UTRs of BNT162b2 (BioNTech) *C3*	DC2.4 cells	*HBB* origin: human β-globin mRNA length: 57 nt *HSPA1A* origin: human heat shock 70 kDa protein 1 mRNA length: 227 nt *Rabb* origin: rabbit α-globin mRNA length: 66 nt *H4C2* origin: histone H4 mRNA length: 42 nt	[163]
Adenoviral tripartite leader sequence (TPL)	UTR strengthened the antigen-specific T-cell response and increased the number of IFNγ-secreting cells in immunized mice	5′ UTR of mRNA-1273 (Moderna)	C57BL/6Cit and I/StSnEgYCit mice	origin: tripartite leader sequence of adenovirus mRNA length: 245 nt	[141]
CYBA	5′ and 3′ UTRs increased protein levels independently and in combination without affecting the half-life of mRNA transcripts.	*DECR1* *GMFG* *MAPBPIP* *MYL6B*	NIH3T3 and A549 cells	origin: human cytochrome *b-245* alpha chain mRNA length: 39 nt	[44, 173]
5′ UTR of S27a-44 transcript of *Rps27a* gene	UTR increased the efficiency of transgene expression *S27a-44′ without the TOP motif improved transgene expression more efficiently than the intact S27a-44.	*CYBA* α-globin *S27a-45* transcript of *Rps27a*	Hep3B and 293T cells	origin: modified UTR from the ribosomal protein S27a mRNA length: different variants	[44]

Table 1 (*cont.*)

Selected 5′ UTR	Advantages	5′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
NASAR	incorporation of NCA-7d as the 5′ UTR and S27a as the 3′ UTR with the functional motif R3U enhanced antigen translation	*CYBA* α-globin	Hep3B and 293T cells	origin: synthetic sequence length: 70 nt	[44]
C3 *CYP2E1*	*in vitro* transcribed mRNAs containing these 5′ UTRs demonstrated improved translational efficiency regardless of the 3′ UTRs present	*ASL* *ALB* *FGA* *ORM1* *HPX* *AGXT* *APOA2*	HepG2 cells	*C3* origin: complement factor 3 mRNA length: 92 nt *CYP2E1* origin: cytochrome *C450* 2E1 mRNA length: 37 nt	[63]
Mouse *COL1A2* *Hoxa9* P4 *Rpl18a* TOP *RBCS1A* TMV TEV HCV chimeric fusion of *hHBB* 5′ UTR with TEV or TOP sequence DEN2 (alone and in combination with DEN2 3′ UTR)	5′ UTRs of these genes ensured higher ribosome loading and translational efficiency than the 5′ UTR of human β globin gene	*hHBB*	HEK293T cells	mouse *COL1A2* origin: mouse Col1a2 gene mRNA length: 137 nt *Hoxa9* P4 origin: mouse Hoxa9 IRES-like element pairing element 4 length: 323 nt *Rpl18a* TOP origin: ribosomal protein 18a with 5′ terminal oligopyrimidine motif length: the length of Rpl18a is 89 nt; the TOP motif is a *cis*-regulatory RNA element that begins directly after the m^7G cap structure and contains the hallmark invariant 5′-cytidine followed by an uninterrupted tract of 4-15 pyrimidines *RBCS1A* origin: *Arabidopsis thaliana* ribulose bisphosphate carboxylase small chain 1A mRNA length: 418 nt TEV origin: Tobacco etch virus mRNA length: 47 nt TMV DEN origin: tobacco mosaic virus UTR and dengue virus UTR fusion length: 68 nt for TMV UTR; 96 nt for DEN UTR	[67]

Table 1 (*cont.*)

Selected 5′ UTR	Advantages	5′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
TMSB10	UTR provided higher levels of gene expression in cultured cells; when administered intramuscularly to mice as a component of mRNA vaccine, the UTR promoted the transgene expression in whole blood, spleen, and liver, leading to enhanced specific humoral and cellular immune responses against the antigen	5′ and 3′ UTRs of *TMSB4X B2M ACTB MTRNR2L2 HLA-DRA HLA-DRB1 FTL FTH1*	DC2.4 cells	origin: thymosin beta 10 mRNA length: 78 nt	[171]
		R27-UTRs	DC2.4 cells BALB/c mice		[171]
TISU (translation initiator of short 5′ UTR)	mRNA with *TISU* UTR demonstrated better expression than mRNAs containing the Kozak sequence only, no 5′ UTR, or eIF4G aptamer as a 5′ UTR *in vivo* translation efficacy was comparable to that of mRNA from the SpikeVax vaccine with 5′ and 3′ UTRs	Kozak sequence eIF4G aptamer mRNA containing 5′ and 3′ UTRs of SpikeVax® vaccine	HEK-293 cells peripheral blood mononuclear cells human T cells C57BL/6 mice	length: minimum 4 nt	[178]

Table 2. Studied 3′ UTRs and their effect on the translational efficiency and mRNA stability

3′ UTR	Advantages	3′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
HBB-HBB	two 3′ UTRs of human β-globin fused head to tail improved the transcript stability and protein yield, increased the number of antigen-specific peptide/MHC complexes on the cell surface, and improved expansion of antigen-specific T cells *in vivo* after injection of transfected bone marrow-derived DCs (BMDCs) in mice	single β-globin 3′ UTR poly-A tail only, no UTR	human immature and mature DCs EL4 cells BMDCs C57Bl/J6 mice	origin: human β-globin mRNA length: 240 nt [163]	[52, 182]

Table 2 (*cont.*)

3′ UTR	Advantages	3′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
HBVpA *BGHpA*	*HBVpA* UTR increased the level of *in vitro* antigen production and antibody titer after immunization of BALB/c mice more efficiently than *BGHpA* and *betapA* UTRs	*HBVpA*, *BGHpA*, rabbit β globin *betapA*	CHO cells BALB/c mice	*HBVpA* origin: hepatitis B virus length: 50-100 nt	[183]
CYBA	incorporation of 3′ and 5′ UTRs enhanced protein translation but did not increase the half-life of mRNA transcripts.	*DECR1* *GMFG* *MAPBPIP* *MYL6B*	NIH3T3 and A549 cells	origin: human cytochrome *b*-245 alpha chain mRNA length: 200 nt	[52, 153]
Rps27a (*S27a*)	UTR increased translational efficiency and protein expression	*CYBA* *TF* *AAT* *HCV* α-globin	Hep3B and 293T cells	origin: human 40S ribosomal protein S27a mRNA length: 170 nt	[44]
NASAR	incorporation of NCA-7d as the 5′ UTR and S27a as 3′ UTR with the R3U functional motif promoted antigen translation	*CYBA* α-globin	Hep3B and 293T cells	origin: synthetic sequence length: 175-190 nt	[44]
mtRNR1-AES *AES-mtRNR1*	UTRs increased protein production and extended mRNA half-life in cells, provided higher splenic expression of luciferase and stronger $CD8^+$ T cell immune response after intravenous vaccination of BALB/c mice, and improved the reprogramming of human foreskin fibroblasts (HFFs) into iPSCs	*HBB* *HBB-mtRNR1* *mtRNR1* *HBB-HBB*	human DCs C2C12 cells HFFs BALB/c mice	*mtRNR1-AES* origin: synthetic sequence length: 300 nt *AES-mtRNR1* origin: synthetic sequence length: 300 nt	[172]
AES-AES *mtRNR-mtRNR*	UTRs provided higher translational efficiency	*AES-HBB* *mtRNR-HBB* mRNA-1273 BNT162b2	DC2.4 cells	*AES-AES* origin: synthetic sequence length: 360 nt *mtRNR-mtRNR* origin: synthetic sequence length: 360 nt	[172]

Table 2 (*cont.*)

3′ UTR	Advantages	3′ UTR used for comparison	*In vitro*/ *in vivo* model	Origin and length	References
DEN2 (alone and in combination with DEN 5′ UTR)	UTR promoted higher ribosome loading and translational efficiency independently; combining it with the DEN 5′ UTR had an additive effect on the translation efficiency and mRNA stability	*HBB* *CYBA* *HBA1*	HEK293T cells	Origin: dengue virus Length: 451 nt [184].	[67]
		rabbit α-globin	Vero cells		[174]
		3′DENΔSL	BHK cells		[184]
TMSB10	UTR provided higher levels of gene expression in cultured cells, as well as in whole blood, spleen, and liver after intramuscular injection to mice, leading to enhanced specific humoral and cellular immune responses against the antigen	5′ and 3′ UTRS of *TMSB4X* *B2M* *ACTB* *MTRNR2L2* *HLA-DRA* *HLA-DRB1* *FTL* *FTH1*	DC2.4 cells	origin: human *TMSB10* mRNA length: 240 nt	[171]
SV40	UTR enhanced transgene expression	*Hsp70*	HepG2, Hep3B, and HEK293 cells	origin: simian virus 40 large T antigen length: different variants	[171]

A recent study reported a series of minimal-length (12-14 nt) synthetic 5′ UTRs that provided higher expression levels than the full-length human α-globin 5′ UTR in cell lines and *in vivo* [186]. Deep learning and massively parallel reporter assay (MPRA) screening of a large set of different UTRs were used to predict the effect of different 5′ UTRs on the translational efficiency to facilitate the design of 5′ UTRs for the optimal protein production [130, 187]. Furthermore, UTR optimization resulted in the optimal combination of endogenous and *de novo* engineered 5′ and 3′ UTRs (named NASARs) that were 5-10 times more efficient than the tested endogenous *CYBA* and α-globin UTRs [44]. Three synthetic 5′ UTRs were identified that significantly outperformed natural 5′ UTRs in [15]. Artificial UTRs were also used to engineer mRNAs for cell reprogramming and vaccine development [154, 188-190]. Based on the above information, the use of endogenous UTRs in combination with the *de novo* design may be the most efficient approach to UTR engineering capable of facilitating the development of mRNA therapeutics [44]. Despite the proven effectiveness of these UTRs, there is a lack of research data on their comparative efficacy, as well as on selection of particular UTRs best suited for specific applications. The absence of standardization and comprehensive control in the selection of UTRs for gene therapeutics and mRNA vaccines is a significant challenge. While some natural UTRs are known to improve the product quality, their use is often based on precedent rather than systematic evidence.

Even if a particular UTR outperforms another UTR with the same coding region, there is no guarantee that this will be true for other coding sequence. This context dependency emphasizes the importance of interpreting the statements about the “best” UTRs with caution. So far, no study has systematically compared all possible UTR combinations, which illustrates a serious gap in understanding the possible approaches for optimizing UTR selection for gene therapies and vaccines. UTRs currently tested in clinical trials are often derived from natural sequences, especially those that have shown functional benefits in past research or vaccine development. Commonly used UTRs include those from the α- and β-globin genes, which are known for their favorable impact on mRNA stability and translational efficiency. These sequences have been successfully used in approved RNA vaccines, e.g., vaccines against COVID-19 [191].

However, the selection process for UTRs remains largely empirical, with many decisions based on a precedent rather than on rigorous comparative studies. The lack of a standardized framework or algorithm for the UTR selection represents a significant

bottleneck in the development of RNA-based therapeutics. As UTRs strongly affect the mRNA stability, translational efficiency, and interactions with regulatory molecules (e.g., proteins, miRNAs), their underexplored potential may hinder innovation in gene therapy and vaccine development.

Systematic strategies are needed to close this gap, such as the high-throughput screening of UTR libraries [15], computational modeling to predict the UTR behavior in different contexts, and establishment of standardized criteria for the UTR design. These efforts could help to develop a more rational and informed approach to the UTR selection, which will ultimately accelerate the progress in the field of mRNA therapeutics.

UTR DESIGN

Although regulatory motifs and the secondary structure of UTRs have no coding function, they significantly influence the translational efficiency and the half-life of transcripts. The design of UTRs for the development of therapeutic molecules is based on the inclusion of regulatory elements from natural sequences flanking highly expressed genes. For example, for the development of mRNA vaccines, Pfizer/BioNTech utilized a 5′ UTR fragment from the human α-globin mRNA in combination with the modified Kozak sequence [191]. Although the understanding of regulatory elements in the UTRs is still incomplete, it has been shown that an efficient 5′ UTR design requires formation of a less structured region upstream of the start codon [68, 192, 193] to promote translation initiation by improving the ribosome accessibility.

It should be mentioned that the results of UTR efficiency tests performed *in vivo* cannot always be extrapolated to the *in vitro* mRNA transcription systems. For example, it was shown that the 5′ UTR of the *CYBA* mRNA provided a higher transcription of the reporter gene *in vivo* compared to its transcription *in vitro* [179]. In other words, the same mRNA can have different properties in cells and cell-free solutions, which can be explained by the presence in living systems of a large number of factors that can interact with mRNA.

To summarize, in both basic and applied research, ORFs are typically combined with UTRs whose ability to increase the translation efficiency has been already demonstrated. In some cases, these constructs are indeed more efficient compared to the wild-type mRNAs, thus expanding the list of UTRs positively affecting the mRNA productivity. However, this approach is not systematic; rather it is a naive enumeration of possible options. New synthetic UTR sequences can be developed using machine learning approaches, which rely on training datasets of sequences with different UTRs, whose efficiency in protein synthesis has been measured. Based on this information, the machine learning methods can predict new UTR sequences outperforming natural UTRs. The quality of such predictions strongly depends on the quality of training datasets. Furthermore, the predicted variants should be validated experimentally. Recent computational approaches to the UTR design are briefly described in Table 3.

EXPERIMENTAL VALIDATION OF COMPUTATIONALLY PREDICTED UTRS

UTR optimization is a crucial step in the development of mRNA-based therapeutics, as UTRs significantly affect mRNA stability and protein production. Several approaches have been developed to analyze and compare the efficiency of UTRs, ranging from traditional reporter gene systems to the state-of-the-art high-throughput methods. Luciferase reporter assays are among the most commonly used techniques for analyzing the UTR functionality. By coupling a reporter gene (*Luc2CP*) to specific 3′ UTR sequences, researchers can evaluate the kinetics of mRNA decay and gain insight into the stability conferred by these UTRs. This assay measures the functional half-life of mRNAs by monitoring the activity of luciferase over time, which ensures a straightforward and reliable assessment of the UTR-mediated stability [172]. Ribosome profiling provides an overall view of translational efficiency by mapping mRNA fragments protected by ribosomes, thus allowing direct measurement of UTR-mediated translational regulation. This technique is especially useful for understanding ribosome stalling, uORFs, and other regulatory elements that influence the translation rate [200]. Another approach is polysome profiling [201], which is based on the separation of translated mRNAs associated with polysomes from untranslated mRNAs using a sucrose gradient.

MPRA enables a high-throughput evaluation of UTR sequences by using synthetic mRNA libraries containing a large number of UTR variants [202]. Each variant is associated with a unique DNA barcode that is incorporated into the reporter transcript. Cells are transfected with the library containing multiple mRNA variants. [203], the most efficient of which are selected by polysome profiling [130, 204] or other methods (e.g., translating ribosome affinity purification [205, 206]) to enrich actively translated mRNAs. Next, RNA sequencing can be performed to identify the variants with a high translational efficiency of the reporter transcript. This approach enables simultaneous interrogation of numerous UTRs to identify the sequence elements that regulate the translational efficiency and stability of mRNA. In addition, MPRA facilitate

Table 3. Computational methods for UTR prediction

Name	Purpose	Approaches to model development	Sequence length limit	Availability	References
FramePool	MRL (mean ribosome loads) prediction for 5′ UTR, analysis of human 5′ UTR variants, prediction of the effects of translation initiation site located upstream of the canonical start codon	one-hot encoding, convolution neural network (CNN) with frame pooling*	any length	https://github.com/Karollus/5UTR	[194]
Optimus 5-prime	MRL prediction for 5′ UTR, development of synthetic 5′ UTR	CNN for MRL prediction; genetic algorithm for evolving new sequences	50 nt	https://github.com/pjsample/human_5utr_modeling	[16, 130]
Fast SeqProp	DNA, RNA, and protein design (strong polyadenylation signals, 5′ UTRs, and enhancers.)	sequence optimization model with VAE-regularization using APARENT, Optimus 5, DragoNN, MPRA-DragoNN, DeepSEA, and trRosetta	varying	https://github.com/johli/seqprop	[195]
UTRGAN	development of synthetic 5′ UTR; MRL and translation efficiency optimization	Deep generative adversarial network (GAN); gradient ascent algorithm for optimization with FramePool and MTtrans 3; one-hot encoding	64-128 nt	https://github.com/ciceklab/UTRGAN	[196]
DEN	development of synthetic 3′ UTRs, splicing regulatory elements, enhancers	Deep exploration networks (DENs)	–	https://github.com/johli/genesis	[197]
FunUV	prediction of functional 5′ UTR and 3′ UTR variants	Gradient boosting decision tree (GBDT)	50 nt for 5′ UTRs and 100 nt for 3′ UTRs	https://github.com/Wangxiaoyue-lab/FunUV	[198]
mRNA2vec	improvement of mRNA stability and protein production	language model transformer architecture-based embedding	for pre-training (using 5′ UTRs and ORFs as input sequences): average length, 459 nt; for the downstream task on 5′ UTR data: average length, 91 nt	–	[199]
PARADE	design of 5′ UTRs and 3′ UTRs for highly stable mRNAs with a programmable cell type specificity	diffusion model; genetic algorithm; random sampling; motif-based design	–	https://github.com/autosome-ru/parade	[120]

Note. * Frame pooling is a method used in CNNs that deal with multiple frames (such as shifts in nucleotide sequences). In this process, the network processes each frame individually, typically by applying global pooling operations (such as max pooling and average pooling) to extract the most important features from each frame. The results of these operations are then combined into a unified representation, preserving individual characteristics of each frame and the overall structure of the input sequence. This enables the network to better understand patterns in genetic data.

Table 4. Challenges and prospects of UTR engineering for gene therapy

Issue	Challenges	Prospects
Precise UTR engineering	development of optimal UTR sequences for specific therapeutic applications is complicated due to the intricate interactions between UTRs and cellular factors which can affect mRNA stability, localization, translational efficiency, and other factors are critical for the control of transgene expression	molecular mechanisms that control UTR function need to be further explored; the development of advanced computational tools and *in vitro* models is essential for the prediction and optimization of UTR sequences; machine learning algorithms and high-throughput screening methods can accelerate the design of UTRs tailored to specific therapeutic outcomes
Long-term safety and efficacy	the long-term safety and efficacy of mRNA-based gene therapies cannot be assured based solely on the *in silico* data, including predicted UTRs; unintended effects, such as immune response or expression of off-target genes, are difficult to predict and pose a risk to patients	rigorous preclinical and clinical testing conducted in compliance with the Good Laboratory Practice and Good Manufacturing Practice standards is essential to ensure the safety of mRNA-based therapies; these standards help to identify potential risks and optimize the therapy protocols; in the context of long-term monitoring in clinical trials, novel technologies in the UTR design enable the prediction of promising therapeutic mRNAs at the beginning of selection process, saving money and resources
Targeted gene delivery	targeted gene delivery to specific tissues or cell types is still a major issue; off-target effects can reduce the therapeutic efficacy and increase the risk of side effects of gene therapies	engineering UTRs with sequences that promote interaction with cellular receptors or transmission vectors could improve tissue-specific targeting; for example, inclusion of tissue-specific miRBSs in the UTRs can restrict gene expression to the desired cell types; this approach would minimize the off-target effects and improve the therapeutic precision
Regulatory and ethical considerations	the regulatory environment for gene therapies is complex, and UTR engineering algorithms and approaches must meet stringent safety and efficacy standards; ethical considerations, such as equitable access and potential misuse, must also be taken into account	collaboration between researchers, regulatory agencies, and industry stakeholders will be crucial to manage the regulatory process; transparent communication about the benefits and risks of mRNA-based therapies will help to gain public trust and ensure ethical implementation of the developed therapies

the investigation of ribosome-dependent and ribosome-independent regulatory mechanisms, which makes it an invaluable tool for the UTR optimization [187, 204]. Each of these experimental approaches has its own strengths and limitations. Reporter assays are simple and specific, but have a limited throughput. MPRA offers a high-throughput screening of UTR variants, but require advanced sequencing technologies. Ribosome profiling and polysome profiling offer deep insights into translation dynamics, however, these methods are technically complex and resource intensive.

Together, these complementary approaches enable systematic assessment of UTRs and provide the basis for rational mRNA design for both therapeutic and research applications. Future advancements, particularly integration of machine learning and computational modeling, promise even greater precision in the selection and optimization of UTRs.

CHALLENGES AND PROSPECTS

Although UTR engineering is a promising approach in gene therapy, a number of issues still remain to be resolved to fully realize its potential. These include precise UTR engineering, the long-term safety and efficacy of gene therapy, targeted gene delivery, and ethical considerations (Table 4).

CONCLUSION

Despite a success in the identification of UTR variants affecting mRNA translation, the rational design of UTRs continues to be a subject of active research. For example, high-throughput screening of 5′ UTR libraries by recombinase-mediated integration has shown that certain UTR variants can significantly increase expression of the transgenic GFP protein in HEK293T cells [15]. In addition, optimization of UTR sequences can be used for controlling the translational capacity of mRNA to ensure the optimal protein synthesis without exceeding the toxicity limits. For example, altering the miRBSs in 3′ UTRs suppressed the expression of toxic transgenes, which in turn reduced the cytotoxicity and increased the AAV vector yield in HEK 293 cells [207]. The modular nature of mRNA allows to combine the selection of UTRs with other approaches aimed to improve gene therapies. For example, a combination of the UTR library screening and codon optimization in the ORF improved the gene therapy for phenylketonuria [208]. In another study [209], the inclusion of an expanded promoter and modification of 3′ UTR elements led to a significant reduction in the hepatic toxicity in the treatment of Rett syndrome in a mouse model. These results highlight the potential of using UTR selection in combination with other gene therapy approaches, such as promoter design [210] and codon optimization [211]. The targeting of therapeutic transgenes to specific tissues also remains a crucial aspect of gene therapy.

Transcriptional (using tissue-specific promoters) and transductional (using viral serotypes) targeting strategies are established methods in the development of tissue-specific therapeutics. There are also significant opportunities to improve the tissue specificity of vector-mediated gene expression through the post-transcriptional regulation, including the miRNA-mediated suppression. Engineered expression cassettes containing miRBSs in the 3′ UTRs allow targeted degradation of transgene mRNAs in cells expressing the corresponding miRNAs [108]. The efficacy of miRNA-regulated transgene expression systems depends on the tissue-specific expression of the selected miRNAs. Recently, miRNAs with suitable expression profiles for a specific tissue have been discovered and described in [106, 212, 213].

The principle of action of mRNA-based gene therapeutics and vaccines includes protein synthesis in a eukaryotic cell. The process of translation largely depends on the UTRs selected for the design of a particular construct. Therefore, the rational design of gene therapeutics and vaccines should involve the stage of UTR selection in order to develop high-efficacy therapeutic agents.

Abbreviations. AAVs, adeno-associated viruses; CCR4-NOT, carbon catabolite repression – negative on TATA-less; DEN2, dengue virus; m^6A, N^6-methyladenosine; mRNA, messenger RNA; miRNA, microRNA; miRBSs, miRNA-binding sites; ORF, open reading frame; PTMs, post-transcriptional modifications; RBPs, RNA-binding proteins; uORF, upstream open reading frame; UTRs, untranslated regions.

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