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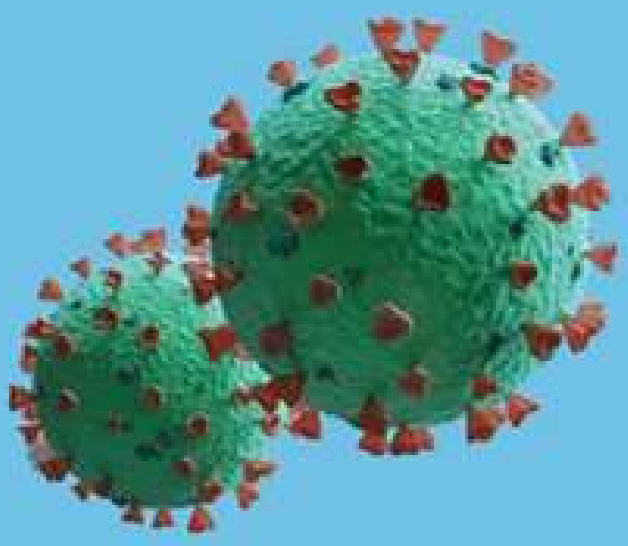
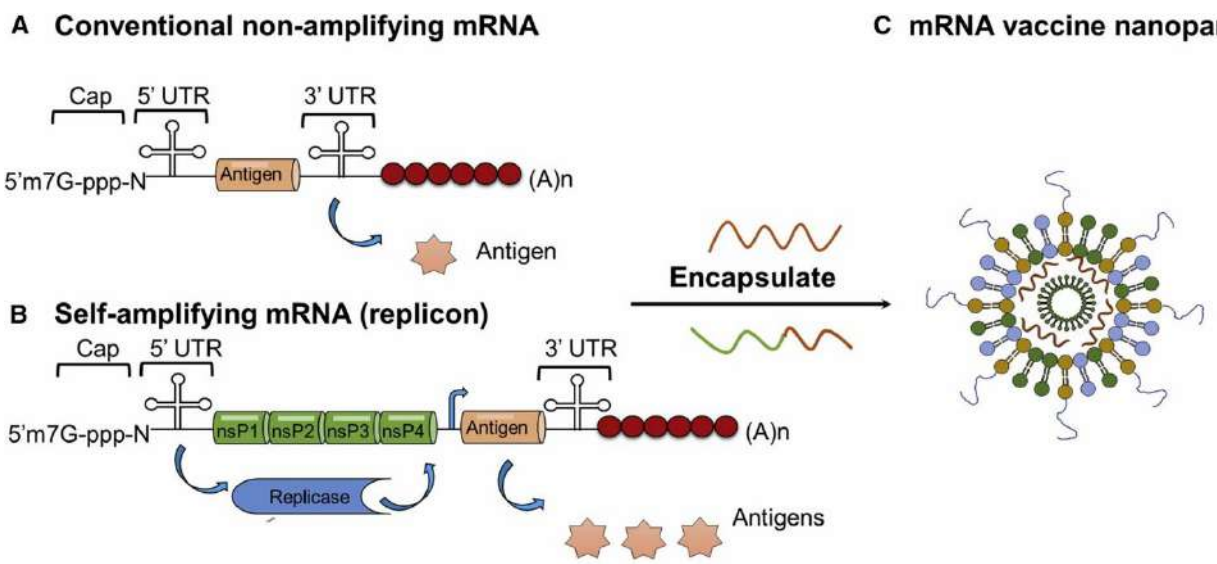
COVID-19 VACCINE: INNOVATIVE PLATFORMS mRNA Vaccines - PART I

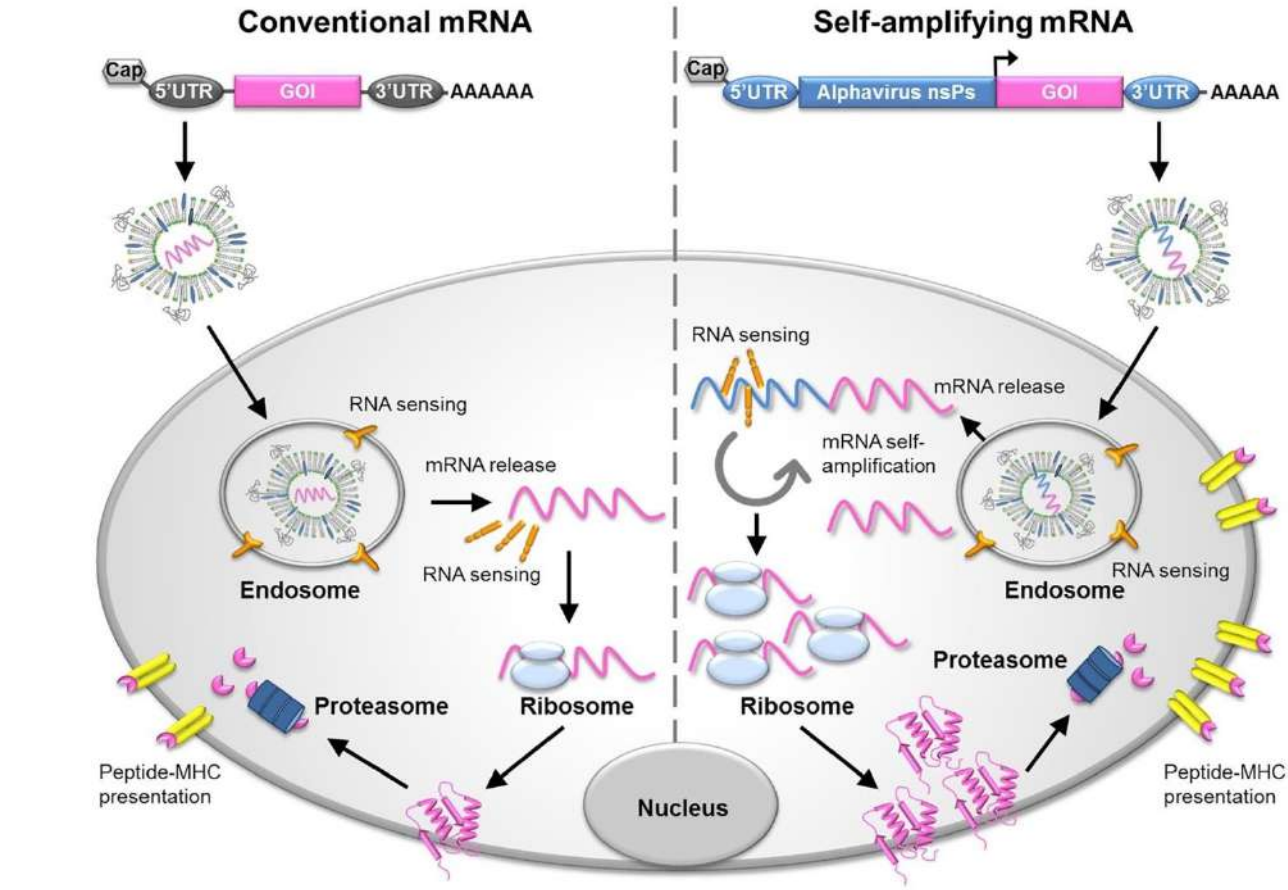
DR. LORETTA BOLGAN

Doctor of Pharmaceutical Chemistry and Technology
Ph.D. in Pharmaceutical Sciences
Scientific advisor





https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453548/
Non-amplifying, self-amplifying mRNA vaccine
(A) Schematic structure of conventional non-amplifying mRNA vaccine.
(B) Schematic structure of the self-amplifying mRNA vaccine (replicone), which contains the sequence-coding antigens and nonstructural proteins that facilitate RNA capping and replication.
(C) An illustration of vaccine mRNA or replicon encapsulated in nanoparticles to improve in vivo performance.



https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(19)30041-3
Schematic representation of mRNA vaccines and mechanism of antigen expression
Conventional mRNA carries the coding sequence of the antigen of interest (GOI) flanked by 5'and 3' UTRs, a 5'-cap terminal structure and a 3' polyA tail. Once transported into the cell and released from the endosome into the cytoplasm, the mRNA is translated immediately. Self-amplifying mRNA is often derived from the genome of positive-sense single-stranded RNA viruses, such as alphaviruses. It encodes both the antigen of interest and the viral nonstructural proteins (nsPs) required for intracellular RNA amplification and high levels of antigen expression. Self-amplifying mRNA can direct its self-amplification to generate RNA intermediates and many copies of subgenomic mRNA coding for the antigen, producing high levels of the encoded antigen. Both conventional and self-amplifying mRNA vaccines require a delivery system for cellular uptake, usually by endocytosis, which is followed by release of the mRNA cargo from the endosome into the cytosol, where translation and protein processing for MHC presentation occur. Once transferred into the cell, the mRNA is almost immediately detected by pattern recognition receptors (PRRs) in the endosome and cytoplasm. PRRs such as Toll-like receptors TLR3, TLR7 and TLR8 are located in the endosome, and cytosolic sensors such as RIG-I, MDA5, PKR and OAS also recognize double-stranded and single-stranded RNAs in the cytoplasm. GOI, gene of interest; MHC, major histocompatibility complex; nsPs, nonstructural proteins.

The mRNA vaccines containing the SARS-Cov-2 spike marketed by Pfizer and Moderna are of the nonamplifying type and contain the basic mRNA structure, with an open reading frame (ORF) coding for the desired antigen.

The main advantages of vaccines with nonreplicating mRNA include:

(1) the relatively small size of mRNA compared with a self-amplifying vaccine (~2-3 kb versus ~10 kb); ;
(2) the absence of additional proteins (compared to the viral system), minimizing the possibility of eliciting unwanted immunogenic interactions with the host. Added to this is a reduced residence time in the cytoplasm, making the possibility of retrotranscribed DNA formation negligible.
(3) are relatively easy to expand and produce, allowing rapid deployment in the event of an outbreak;
(4) easy engineering of sequences to improve vaccine performance and minimize side effects.

The biggest obstacle to the use of mRNA vaccines is the need for intracellular delivery.
Chemical modifications and sequence engineering have improved both the translation and shelf life of synthetic mRNA vaccines, while mRNA delivery has been achieved through the use of lipids (liposomes) or polymer-based materials.[11]

[11] Kowalski PS, Rudra A, Miao L, Anderson DG.
Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery.
Mol Ther. 2019;27(4):710-728. doi:10.1016/j.ymthe.2019.02.012
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TECHNOLOGICAL DEVELOPMENT

From a technological point of view, as mentioned above, mRNA vaccines were developed to overcome the limitations of traditional vaccines, compared with which they have proven to have many specific advantages.

First of all, mRNA can theoretically meet all the requirements to encode genetic information and express all types of proteins.
The efficiency for vaccine development can be optimized by modifying the mRNA sequence, which is a more cost-effective way than other types of modification to produce a new vaccine antigen. [12]
In addition, the production and purification processes of an mRNA vaccine are quite similar, despite the different encoded antigens, so it is possible that they may be maintained or even standardized to develop other mRNA-like vaccines. [13]
By modifying the mRNA sequence and delivery system, the expression activity and *in vivo* half-life of mRNA can be effectively regulated. [14]
The use of *in vitro* transcription facilitates the time- and cost-saving production of mRNA vaccines and can also avoid contamination by proteins and viruses from cell cultures.

Second, mRNA has self-adjuvanting properties that activate strong and long-lasting adaptive immune responses through the secretion of tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and other cytokines by immune cells[15] , whereas polypeptide- and protein-based vaccines require additional adjuvants to achieve a similar goal. [16]

Third, compared with DNA vaccines (both nucleic acid and vector), mRNA vaccines can express target proteins more efficiently because of their expression in the cytoplasm without entering the nucleus. In addition, the chemical constitution of the mRNA sequence, due to the lack of CpG islands, reduces the risk of integration into the host DNA genome and immune reaction.

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https://pubmed.ncbi.nlm.nih.gov/28701102/

Finally, mRNA remains active only transiently, and is completely broken down through physiological metabolic pathways. [17]

Design strategies for mRNA vaccines against SARS-COV-2

The design of mRNA vaccines might seem fairly trivial because of their simple vaccine mechanism of action: following administration of vaccine mRNA encoding for a target antigen, cells take up the mRNA, translate it into proteins *in situ, so* that the individual's immune system is able to induce a robust adaptive immune response against the target protein. However, the current mRNA vaccine design process requires important considerations of mRNA modifications to reduce reactogenicity and optimize protein expression, proper selection of target antigen, and optimal formulation to enable efficient delivery.[18]

Changes in mRNA

The first aspect to consider in developing mRNA vaccines is that unmodified mRNA itself is not ideal for use.
Indeed, mRNA is both extremely labile and rapidly degraded under unfavorable conditions. Moreover, it is highly immunogenic and capable of activating a variety of pathogen-associated molecular pattern sensors.
In an effort to improve half-life, translatability, and safety, Karikó et al. tested various natural modifications to nucleosides in mRNA molecules, including pseudouridine, 5-methylcytidine, N6-methyladenosine, 5-methyluridine, and 2-thiouridine[19] .
Of these variants, they found that incorporation of N1-methyl-pseudouridine (m1Ψ) instead of uridine led to a 10-fold increase in translation compared with unmodified mRNA. Furthermore, they were able to show that mRNA molecules possessing this modification did not activate pathogen-associated molecular pattern-sensing mechanisms such as toll-like receptors (TLRs) or retinoic acid-inducible gene I (RIG- I). This is critical to avoid excessive inflammation, which could result in undesirable side effects of the vaccine.
For these reasons, many candidates, including the two recently licensed mRNA vaccines mRNA-1273 and BNT162b2, have adopted mRNA modification with m1Ψ in their vaccine design. [20]

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[17] Sahin U, Karikó K, Türeci Ö.
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Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability.
Mol Ther. 2008 Nov;16(11):1833-40. doi: 10.1038/mt.2008.200. Epub 2008 Sep 16.
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SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness.

Antigen selection

In antigen selection for an mRNA vaccine, it is essential to choose a target that is immunogenic and capable of eliciting a protective immune response.
Of the multiple epitopes of SARS-CoV-2, the spike glycoprotein (S) is the commonly selected target for COVID-19 vaccine development[21] , as it is the major surface protein of SARS-CoV-2 and mediates viral entry by binding to the angiotensin-converting enzyme 2 (ACE2) receptor in host cells[22] .

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Safety and Immunogenicity of SARS-CoV-2 mRNA-1273 Vaccine in Older Adults.
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Annette B. Vogel, et al
A prefusion SARS-CoV-2 spike RNA vaccine is highly immunogenic and prevents lung infection in non-human primates
bioRxiv 2020.09.08.280818; doi: https://doi.org/10.1101/2020.09.08.280818
https://www.biorxiv.org/content/10.1101/2020.09.08.280818v1.full.pdf

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COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses.
Nature. 2020 Oct;586(7830):594-599. doi: 10.1038/s41586-020-2814-7. Epub 2020 Sep 30. Erratum in: Nature. 2021 Feb;590(7844):E17.
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N Engl J Med. 2020 Dec 17;383(25):2439-2450. doi: 10.1056/NEJMoa2027906. Epub 2020 Oct 14.
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https://doi.org/10.1038/s41586-020-2798-3

[22] Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS.
Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation.
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Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein.
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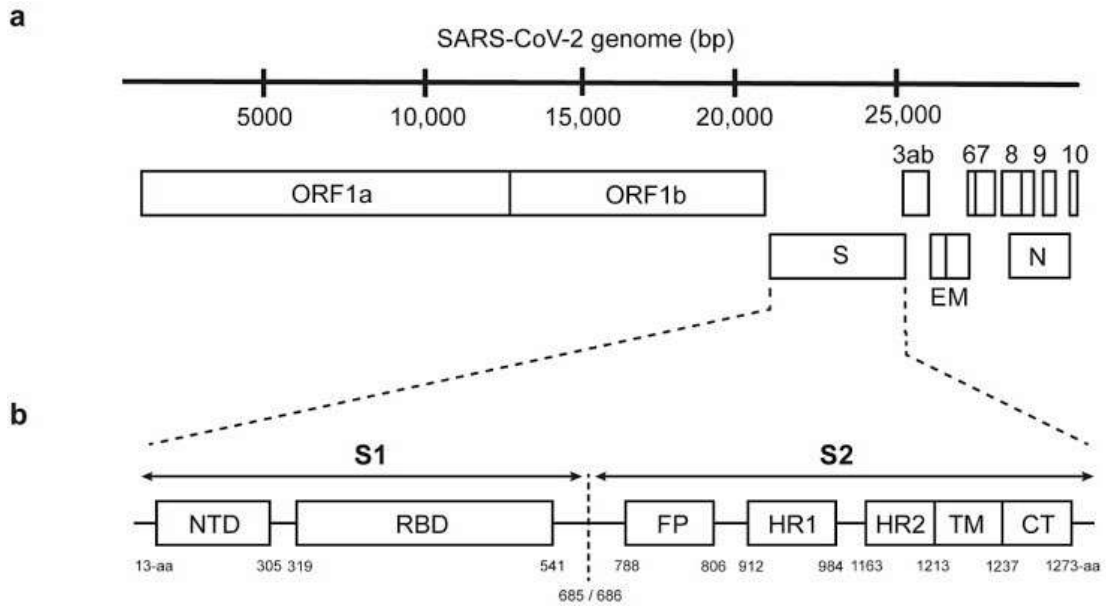
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102599/

Letko M, Marzi A, Munster V.
Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses.
Nat Microbiol. 2020 Apr;5(4):562-569. doi: 10.1038/s41564-020-0688-y. Epub 2020 Feb 24.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7095430/

Spike is a class I viral fusion glycoprotein consisting of a receptor binding subunit (S1) and a fusion subunit (S2) joined by a furin cleavage site unique to this coronavirus[23] .
The spike is cleaved posttranslationally at this furin site. However, the S1 and S2 subunits remain associated until the spike is bound to the ACE2 receptor via the receptor binding domain (RBD).



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7923282/
The S glycoprotein contains two subunits, namely the N-terminal S1 and the C-terminal S2. The length of the SARS-CoV-2 S glycoprotein is 1273 amino acids (aa), arranged in sequence with a 13-aa signal peptide located at the N-terminus followed by the S1 subunit (residues 14-685) and the S2 subunit (residues 686-1273). Within the S1 subunit, there is an N-terminal domain (residues 14-305) and a receptor binding domain (RBD; residues 319-541), while the fusion peptide (FP; residues 788-806), heptapeptide repeat sequence 1 and 2 (HR1; residues 912-984 and HR2; residues 1163-1213), the transmembrane domain (TMD; residues 1213-1237) and the cytoplasmic domain (residues 1237-1273) form the S2 subunit.[24]

[23] Kirchdoerfer RN, Cottrell CA, Wang N, Pallesen J, Yassine HM, Turner HL, Corbett KS, Graham BS, McLellan JS, Ward AB.
Pre-fusion structure of a human coronavirus spike protein.
Nature. 2016 Mar 3;531(7592):118-21. doi: 10.1038/nature17200.
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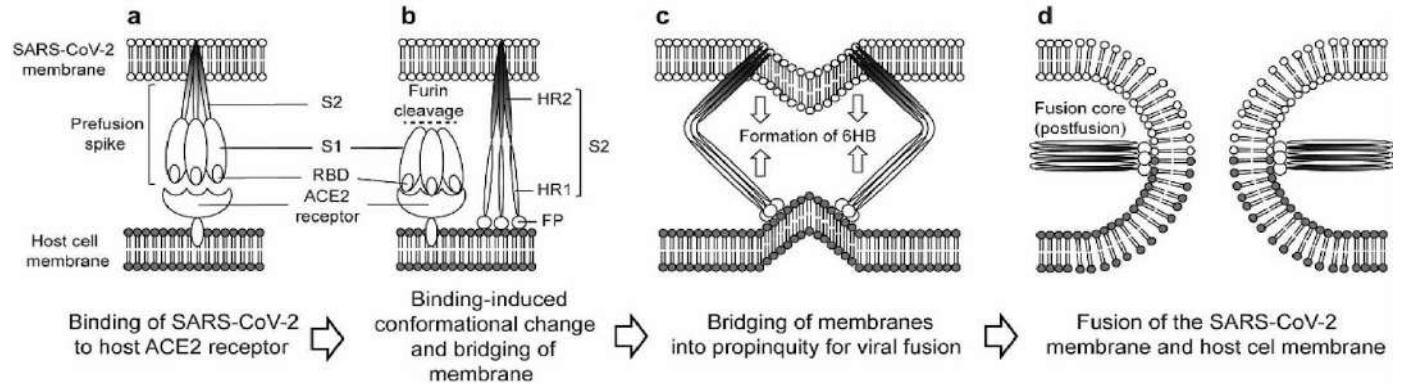
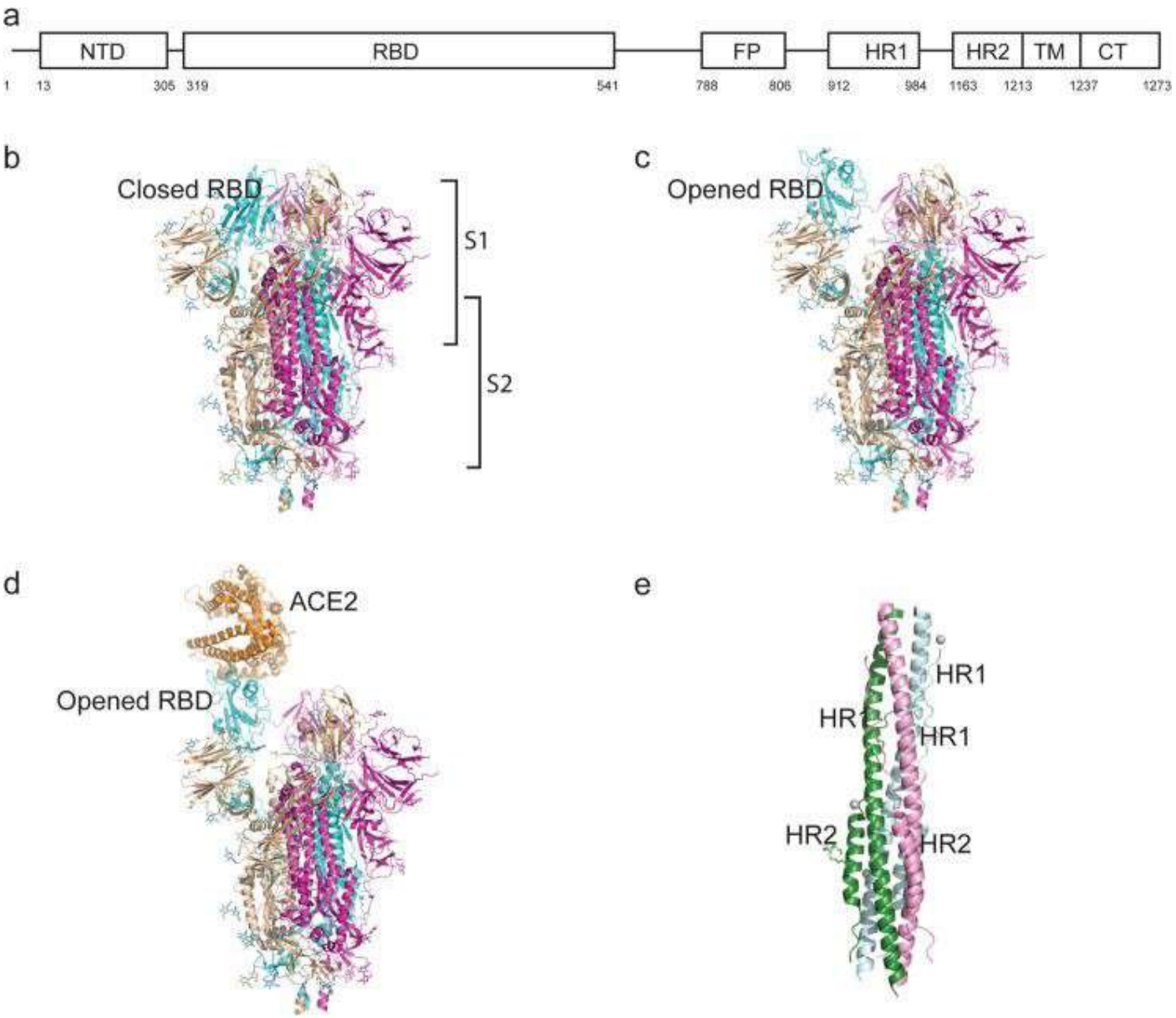


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7923282/

Schematic representation of **(a)** binding of the RBD pre-fusion spike of SARS-CoV-2 (S) to accommodate the ACE2 receptor, **(b)** cleavage of the S protein into the S1 subunit and S2 subunit by furin, resulting in the fusion peptide (FP) of S2 exposed and implanted in the membrane of the target cell, **(c)** formation of the 6-helix bundle (6HB) connecting the membranes in close proximity for viral fusion, and **(d)** three HR1 and HR2 combining to form the fusion core (post-fusion) to fuse the viral-host membrane.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7396720/

Structure of the SARS-CoV-2 S protein. **a)** Schematic representation of the spike of SARS-CoV-2. **b) - c)** Closed and open state of the S protein RBD. **d)** The S protein binds to ACE2 with open RBD in the S1 subunit. **e)** The six-helix structure formed by HR1 and HR2 of the S2 subunit.

Information gathered from previous work with similar fusion glycoproteins has shown how important it is to use pre-fusion stabilized proteins that preserve neutralization-sensitive epitopes for the development of effective vaccines[25] .
To stabilize the S protein, several strategies have been adopted. The approach used in the licensed mRNA- 1273 and BNT162b2 vaccines is the introduction of a mutation in which amino acids 986 and 987 are replaced with prolines (S-2P), which stabilize the S glycoprotein in the pre-fusion conformation but still allow cleavage of S1 and S2 subunits.

Vehiculation of mRNA

Although naked mRNA can be injected directly for immunization, this method of delivery is rather inefficient. In fact, mRNA molecules must be able to penetrate through the lipid membrane of a cell to reach the apparatuses required to translate transcripts into proteins.
Therefore, delivery methods that facilitate cytosolic localization of mRNA vaccines are important to achieve efficient translation into protein.
The advent of lipid nanoparticle (LNP) encapsulation was a turning point in the development of mRNA vaccines, as they can effectively deliver mRNA *in vivo*[26] .
When injected intramuscularly, mRNA-LNPs can be phagocytosed and rapidly translated into antigen-presenting cells at both the injection site and draining lymph nodes, thus promoting the initiation of adaptive immune responses[27] . In addition, LNPs can protect mRNA from degradation by nucleases.

Although the precise composition of LNPs used by many vaccine developers is confidential information, it is known that LNPs contain a combination of ionizable cationic lipids, cholesterol, phospholipids, and PEGs that self-assemble into ~100 nm nanoparticles that encapsulate mRNA. [28]

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[25] Pallesen J, et al
Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen.
Proc Natl Acad Sci U S A. 2017 Aug 29;114(35):E7348-E7357. doi: 10.1073/pnas.1707304114. Epub 2017 Aug 14.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6936610/

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J Control Release. 2015 Nov 10;217:345-51. doi: 10.1016/j.jconrel.2015.08.007. Epub 2015 Aug 8.
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Among candidate vaccines in clinical trials, LNPs are the standard method used to deliver mRNA vaccines to participants.

Synthesis and optimization of mRNA [29]

Functional synthetic mRNA can be obtained by *in vitro* transcription of a cDNA template, typically plasmid DNA (pDNA), using a bacteriophage RNA polymerase. [30]
Thus, pDNA preparation is the first step in mRNA production.
The crude pDNA contains traces of bacterial genomic DNA and three forms of pDNA (supercoiled, relaxed circular, or linear) in varying proportions, so the preparation of pure and standardized pDNA, as required for a vaccine, is challenging.
However, bacterial DNA residues and pDNA heterogeneity are not a problem if linearized pDNA is transcribed using bacteriophage RNA polymerase,[31] because all residual DNA is removed during subsequent production steps (see below).

To date, in vitro mRNA transcription technology has been optimized, and the most popular method uses T3, T7 or SP6 RNA polymerase and linear DNA (linearized plasmid DNA or synthetic DNA prepared by PCR) for mRNA synthesis.
There are some basic structural elements that are required to maintain functional mRNA, including a 5' cap (5'-cap), that is, a 7-methyl-guanosine residue joined to the 5' end via a 5'-5' triphosphate,[32] an untranslated 5' region (5'-UTR), an open reading frame (ORF) region, a 3' untranslated region (3'-UTR) and a poly(A)-based tail. [33]

[29] Schlake T, Thess A, Fotin-Mleczek M, Kallen KJ.
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In Vitro Transcription of Long RNA Containing Modified Nucleosides. In: Rabinovich P.M.,
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Weng Y, Li C, Yang T, Hu B, Zhang M, Guo S, Xiao H, Liang XJ, Huang Y.
The challenge and prospect of mRNA therapeutics landscape.
Biotechnol Adv. 2020 May-Jun;40:107534. doi: 10.1016/j.biotechadv.2020.107534. Epub 2020 Feb 21.
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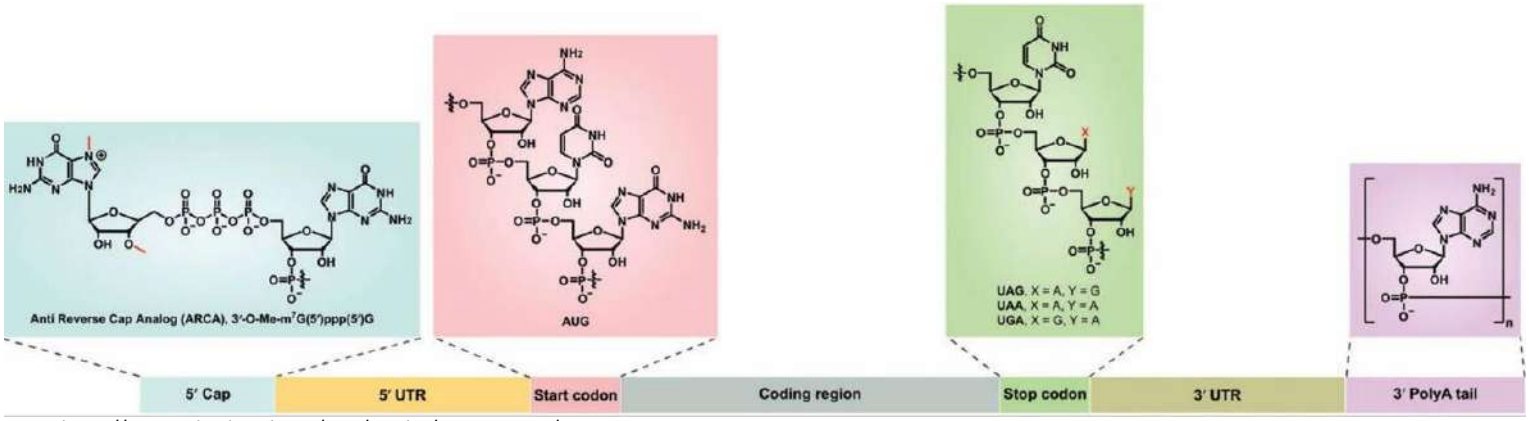
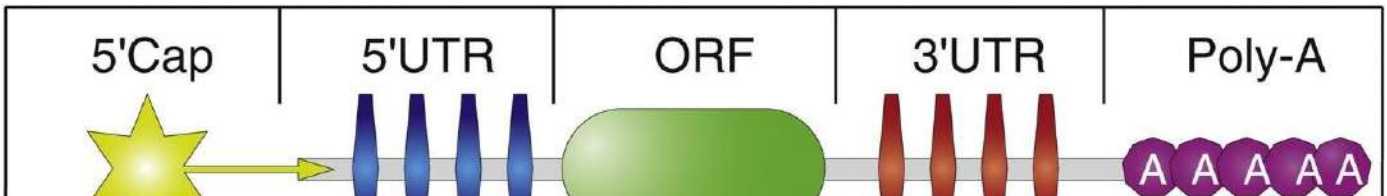
Bardwell VJ, Zarkower D, Edmonds M, Wickens M.
The enzyme that adds poly(A) to mRNAs is a classical poly(A) polymerase.
Mol Cell Biol. 1990 Feb;10(2):846-9. doi: 10.1128/mcb.10.2.846.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC360888/pdf/molcellb00038-0422.pdf



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6443240/

Accordingly, a pDNA mold for *in vitro* transcription must contain at least one bacteriophage promoter, one ORF, possibly one poly(d (A/T)) tail transcribed into poly(A), and a single restriction site for plasmid linearization to ensure defined transcription termination.

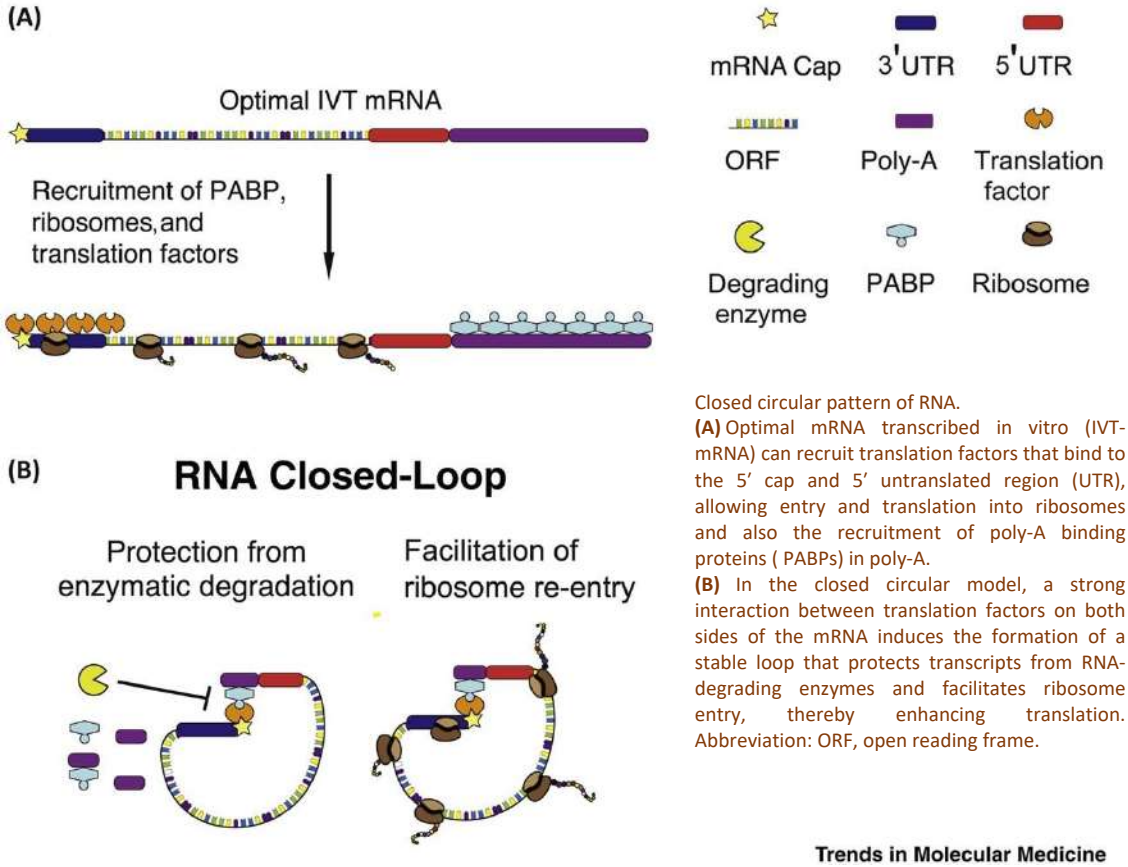


Biological relevance				
Cap -Eukaryotic modification -Important for translation initiation, mRNA stability, nuclear export. -When suboptimal, recognized as PAMPs by the innate immunity. -RNA Closed-Loop.	**5' UTR** -Recognition by translation machinery. -Recognized and scanned by ribosomes. -Important for mRNA translation and stability. -RNA Closed-Loop.	**ORF** -Sequence encoding the gene of interest (GOI), in mRNA vaccine encodes the antigen.	**3'UTR** -Important for translation initiation and mRNA stability.	**Poly A** -Important for mRNA stability. -Recognition by Poly-A binding proteins (PABP) and recruitment of translation factors. -Important for translation initiation (RNA Closed-Loop).
Region optimization				
-Natural Cap-1 to avoid PRR recognition and enhance translation. -Enzymatic capping for higher capping efficiency.	-Inclusion of Kozak sequence. -No strong secondary structures. -No other start codon. -Polysome profiling to count the ribosome loading in sequences *in-silico*.	-Codon optimization increases translation. -Low optimal codons may be important for adequate folding.	-Optimal sequences derived from highly stable mRNA (β-Globin). -2x copies in tandem -RNA Closed-Loop	-Poly-A sequences of 120 units. -Adding a poly-U sequence, providing a dsRNA in the poly-A, increases adjuvant effect.
Whole molecule optimization				
-Avoiding binding sites of miRNAs present in the target cells. -Uridine depletion to avoid recognition by innate immunity. -Production at high temperature (50°C) using a thermostable polymerase and/or low Mg^{2+} concentration, to decrease dsRNA levels. -mRNA purification using HPLC to decrease dsRNA amount. -Avoidance of highly stable and long secondary structures that could activate PRRs.				

Trends in Molecular Medicine

https://www.cell.com/trends/molecular-medicine/fulltext/S1471-4914(19)30244-8
Design of mRNA, from its domain organization to its sequence optimization.
A schematic representation of the major 5 ' cap, 5 ' untranslated region (UTR), open reading frame (ORF), 3 ' UTR and polyadenylation (Poly-A) regions of any mRNA is presented at the top of the figure. Below this structure are the main functions of these regions, their potential interaction with pattern recognition receptors (PRRs), and possible optimizations. Abbreviation: PAMPs, pathogen-associated molecular patterns.

The linearized pDNA template is transcribed into mRNA in a mixture containing recombinant RNA-polymerase (T7, T3 or SP6) and nucleoside triphosphates.
To obtain a closed mRNA, a cap analogue such as m7G(5')-ppp- (5')G dinucleotide (called "regular cap analogue") can be included in the reaction. [34]
If the cap analog is in excess of GTP, transcription begins with the cap analog instead of GTP, producing a closed mRNA.
Alternatively, the cap can be added enzymatically after transcription.



Closed circular pattern of RNA.
(A) Optimal mRNA transcribed in vitro (IVT-mRNA) can recruit translation factors that bind to the 5′ cap and 5′ untranslated region (UTR), allowing entry and translation into ribosomes and also the recruitment of poly-A binding proteins (PABPs) in poly-A.
(B) In the closed circular model, a strong interaction between translation factors on both sides of the mRNA induces the formation of a stable loop that protects transcripts from RNA-degrading enzymes and facilitates ribosome entry, thereby enhancing translation. Abbreviation: ORF, open reading frame.

https://www.cell.com/trends/molecular-medicine/fulltext/S1471-4914(19)30244-8

A poly(A) tail can also be added after transcription if it is not provided by the pDNA template. [35]
After transcription, the pDNA mold and contaminating bacterial DNA are digested by DNase.

Capping of mRNA

The 5' cap of mRNAs is characteristic of eukaryotic mRNA and consists of the addition of an N7-methylated guanosine to the first nucleotide of these molecules. [36]

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Recognition of cap structure in in vitro splicing of mRNA precursors.
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Jang SK, Paek KY.
Cap-dependent translation is mediated by 'RNA looping' rather than 'ribosome scanning'.
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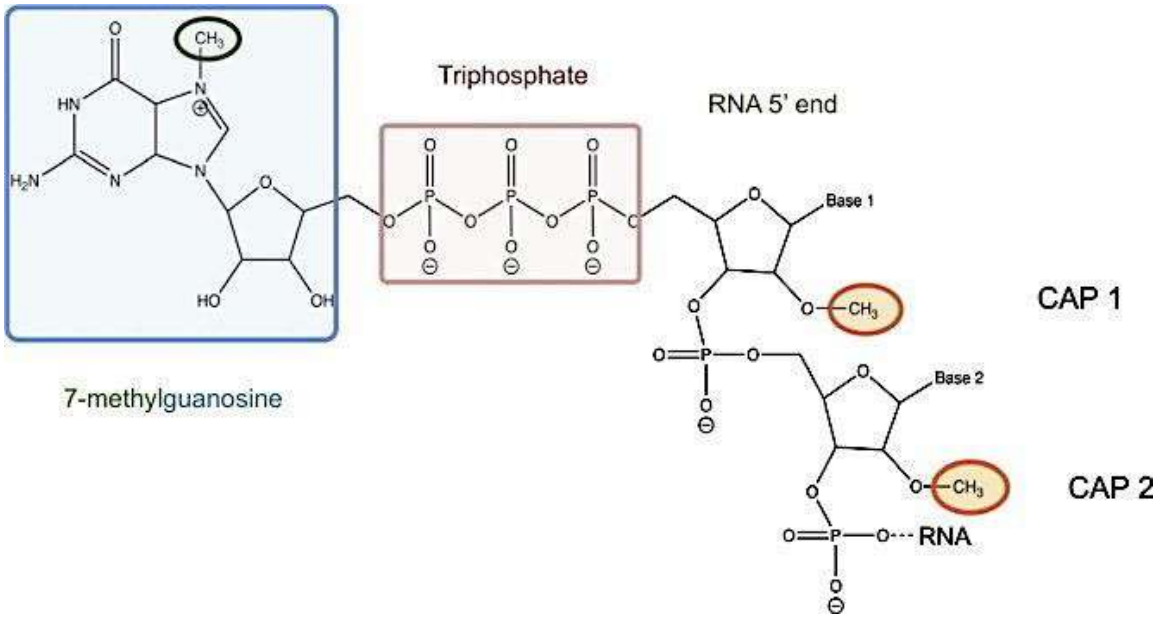
In addition to its functions in pre-mRNA splicing, polyadenylation, nuclear export, and protection against exonucleases, other key aspects of the 5' cap are important to the production of the vaccine construct.
First, vaccine mRNAs should be recognized as molecules of the self.
The 5' cap can be present in different conformations in mRNAs, and a natural CAP-1-like structure is required.
In fact, mRNAs with abnormal capping (CAP-0) or without capping (5'ppp or 5'pp) are recognized by PRRs[37] , such as RIG-I and IFIT, which trigger the production of type I IFNs and their destruction.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7114304/
The RNA cap structure: the cap consists of a 7-methylguanosine (blue box) connected to the 5' nucleoside of the messenger RNA chain via a 5' - 5 ' triphosphate bridge (pink box). The methyl group of guanosine at its N-7 position is surrounded in green, and the 2'-O methyl group of the first and second nucleotide residues forming the cap-1 and cap2 structures, respectively, is surrounded in light orange.

In the conventional route, a cap structure is added to the nascent 5'-triphosphate mRNA in a series of reactions.
The 5'-triphosphate is first hydrolyzed by an RNA 5'-triphosphatase (RTPase).
A guanyltransferase (GTase), also called "capping enzyme," adds cap structure in the form of
Of a guanosine 5'-monophosphate with 5' - 5 ' orientation.
The cap is then methylated at the N-7 position of its guanine by an RNA-cap guanine N-7-methyltransferase (N-7 MTase).
This generates minimal cap-0 (m7GpppN...), which is found in metazoans and lower eukaryotes.

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The viral RNA capping machinery as a target for antiviral drugs.
Antiviral Res. 2012;96(1):21-31. doi:10.1016/j.antiviral.2012.07.007
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Inhibition of translation by IFIT family members is determined by their ability to interact selectively with the 5'-terminal regions of cap0-, cap1- and 5'ppp- mRNAs.
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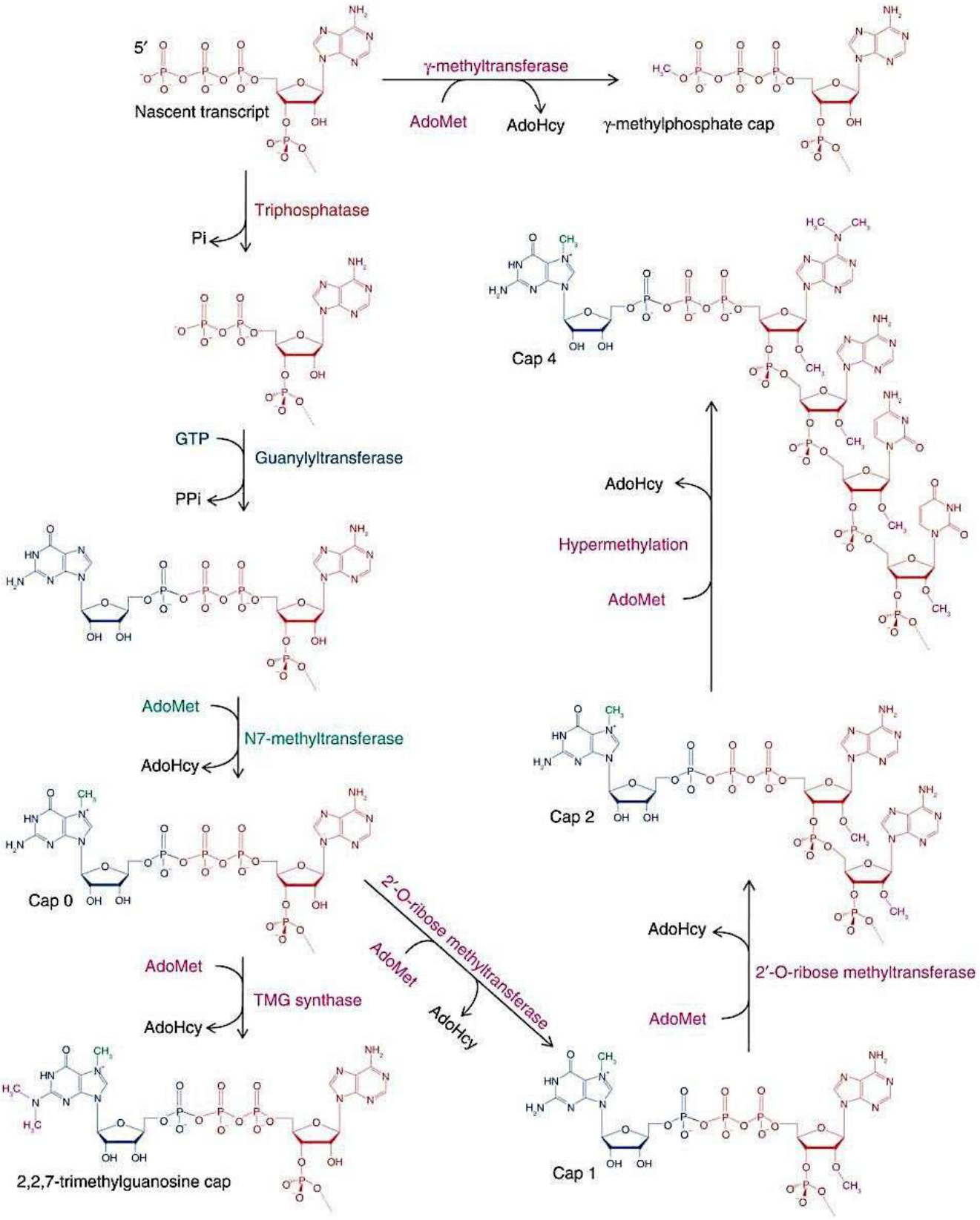
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3950709/

In higher eukaryotes, further methylation by ribose 2'-O-methyltransferase (2'-O MTase) occurs at the 2' position of the riboses of the original transcript to produce mainly cap-1 structures (m7GpppNmN ...) but also cap-2 (m7GpppNmN ...).[38]



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Enzymology of RNA cap synthesis.
Wiley Interdiscip Rev ANN. 2010;1(1):152-172. doi:10.1002/wrna.19
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3962952/
Structures and synthesis of RNA caps. Cap 0 or m7G caps (GMP colored blue) are formed in sequential steps by three enzymatic activities acting on the 5' triphosphate end (colored red) of nascent transcripts. Transfer of one methyl group (colored green) from S-adenosylmethionine (AdoMet) completes the synthesis of cap 0. Transfer of two methyl groups (colored in magenta) from S-adenosylmethionine (AdoMet) is required to form the TMG cap. Cap structures 1 and 2 require methylation (colored magenta) of cap 0 at ribose 2′-O of the first and second nucleosides, respectively. The structure of cap 4 is generated by six cycles of methylation (colored magenta). The first three methylation cycles (color magenta) occur at two positions on the base and ribose of the first nucleoside of the primary transcript. The next three methylation cycles (color magenta) occur at the 2′-O ribose positions of the next three nucleosides. The γ-methylphosphate cap is formed by the transfer of a methyl group (colored magenta) from AdoMet to the γ-phosphate of the primary transcript.

Because of its coupling to RNA transcription, capping is primarily a nuclear process, although some RNA re-capping events are suspected to occur in the cytoplasm[39] .
Viruses generally replicate in the cytoplasm, resulting in a time window during which viral RNAs are synthesized but not yet added to the cap.

The coevolution of viruses and cells has generated a number of cellular pathways and proteins involved in detecting the presence of viral RNAs.
The absence of RNA-cap and the presence of double-stranded RNA are strong signals of viral infection. These RNA species, alone or together, are detected as "non-self" RNAs by cellular sensors that trigger an innate cellular immune response. [40] Viruses have developed numerous strategies to escape detection, including rapid and efficient capping of viral RNA.

The cap structure plays important functions in mRNA translation through the recruitment of translation initiation factors and probably also by facilitating the formation of closed circular mRNA[41] .
Therefore, in the context of the vaccine construct, it is important to achieve maximum efficiency in mRNA production with CAP-1 to avoid excessive activation of innate immunity through the remaining products without cap or with inadequate caps.

Untranslated regions

Untranslated regions (UTRs) are important regulators of mRNA degradation and translation efficiency by RNA-binding proteins.
The use of UTRs of α-globin or β-globin from Xenopus laevis or humans has been the historical standard approach in mRNA vaccination because of their high stability.
However, the performance of UTRs depends on the species, cell type and cell state.
Therefore, any mRNA vaccine must define which UTR sequences are most relevant in the target cells for its strong expression.
Recent approaches for improving UTR to achieve high mRNA expression and stability in target cells/tissues are discussed below.

5’-UTR

Several features of the 5'-UTRs that influence mRNA translation have been described.

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Cap-dependent translation is mediated by 'RNA looping' rather than 'ribosome scanning'.
RNA Biol. 2016;13(1):1-5. doi:10.1080/15476286.2015.1107700
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For example, a canonical start codon (AUG) and non-canonical start codons (AUG and CUG) should be avoided in the 5'-UTR.
Another feature that negatively affects mRNA translation is the presence of highly stable secondary structures, which prevent ribosome recruitment, scanning, and initiation of codon recognition. Apart from these sequence requirements, little is known about how to optimize 5'-UTR sequences.
Bioinformatics is a promising tool for predicting the efficiency of mRNA translation based on the characteristics of 5'-UTR sequences, such as the presence of secondary structures. [42]

3'-UTR

The 3'-UTR is normally considered a concentrated region of unstable factors, so avoiding unstable sequences and introducing stable elements during 3'-UTR synthesis can increase the stability of the mRNA and expand its half-life. [43]
AU- and GU-enriched sequences are exploited for this,[44] as the 3'-UTR determines protein levels through the regulation of mRNA stability and translation mediated largely by AU- and miRNA-rich elements (see below). [45]
In addition to using stable mRNA sequences, protein expression could be improved by adding 3'-UTR sequences twice in tandem. [46]

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Stability analysis of chemically modified mRNA using micropattern-based single-cell arrays.
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Improving mRNA-Based Therapeutic Gene Delivery by Expression-Augmenting 3' UTRs Identified by Cellular Library Screening.
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Blood. 2006 Dec 15;108(13):4009-17. doi: 10.1182/blood-2006-04-015024. Epub 2006 Aug 29.
https://ashpublications.org/blood/article/108/13/4009/6595/Modification-of-antigen-encoding-RNA-increases

A recently developed method to identify 3'-UTR sequences t h a t stabilize mRNA uses progressive enrichment of synthetic mRNA of longer half-life through several rounds [transfection into human dendritic cells (DCs), inhibition of transcription and mRNA purification] of reverse transcriptase (RT)-PCR. This method also identifies a negative correlation between the number of miRNA binding sites* in DCs and the half-life of mRNA.
Therefore, UTR sequences could be controlled to minimize the number of miRNA binding sites in APCs, as these molecules promote mRNA degradation.
It is also possible to incorporate binding sites for miRNAs into the mRNA sequence to d e c r e a s e antigen expression in nontargeted tissues. [47]

** MicroRNAs (miRNAs) are small noncoding RNAs (21 to 25 nucleotides) that negatively regulate gene expression at the post-transcriptional level, and exert their role through recognition and pairing to specific mRNA sequences*

MicroRNAs: their genes and targets
microRNA Structure and Function

The 3'-UTRs also enable local translation through regulation of mRNA localization[48] .
New insights have been gained that the length of the 3'-UTR can be regulated by alternative cleavage and polyadenylation (APA)[49] , as well as that 3'-UTRs can be detached and act as long noncoding RNAs independent of the coding region.[50]

Orlandini von Niessen AG, Poleganov MA, Rechner C, et al.
Improving mRNA-Based Therapeutic Gene Delivery by Expression-Augmenting 3' UTRs Identified by Cellular Library Screening.
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https://www.cell.com/molecular-cell/pdfExtended/S1097-2765(15)01005-9

Finally, 3'-UTRs have been found to mediate protein-protein interactions (PPIs), with a spillover effect on protein complex formation, protein localization and functions[51] and regulate gene expression through binding of RNA-binding proteins (RBPs). [52]

RBPs bind to the *cis* elements of 3'-UTRs and mediate the functions of 3'-UTRs through the recruitment of effector proteins.

The cellular state determines the RBPs that are able to access the 3'-UTRs at any given time, therefore, the functions of the 3'-UTRs can only be evaluated in the context of their RBPs with which they are associated.

The composition of RBPs bound to a 3'-UTR at any given time is dynamic and can change depending on the local environment, for example, through the addition of post-translational modifications (PTMs), local expression of other RBPs with which they bind to enable functional specificity in vivo, and interactions with membranes and cytoskeleton filaments[53] .

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Widespread Differential Expression of Coding Region and 3' UTR Sequences in Neurons and Other Tissues.
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mRNA transport meets membrane traffic.
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https://pubmed.ncbi.nlm.nih.gov/25110341/

Singh G, Pratt G, Yeo GW, Moore MJ.

Finally, the binding of RBPs is also influenced by the structure of secondary and tertiary RNA, which regulates the accessibility
of the 3'-UTR[54] .

Poly-Adenylated Tail

One of the last steps in mRNA transcription is the addition of a poly-A tail, which is usually about 250 units long in Metazoa.
However, recent advances in RNA sequencing cast doubt on this dogma: highly expressed genes, thus naturally occurring mRNAs with strong translation efficiency, rich codons and optimal ribosomal association, contain the shortest poly-A tails. [55]
According to the pruning ("shortening") model, the poly-A-binding protein (PABP) proximal to the 3'-UTR in stable mRNAs is tightly associated with the 5'-cap via the translation initiation factors eiF4G and eiF4E, promoting the formation of a closed circular structure (closed-loop model* see figure p. 75) and efficient translation.

** The most sophisticated model for translation initiation currently is the so-called **"closed-loop" model**, in which a circularization of mRNA is mediated by proteins that bind to the 5'-cap and 3'-poly (A).*
Depending on differential interactions, this event may cause translational stimulation or repression.

The multiple protein-protein and protein-RNA interactions present in the closed circular structure prevent further transcript deadenylation and mRNA degradation.

https://escholarship.org/content/qt6cp950hv/qt6cp950hv_noSplash_03fa99c2eb7b8090b5eb49be4f901351.pdf?t=oil1l7
Synthesis, processing and translation of mRNAs. The mRNAs are transcribed from RNA Pol II into the nucleus. Nascent transcripts go through a co-transcriptional maturation process involving 5'-capping, intron splicing, 3' cleavage, and polyadenylation. Two RNA-binding complexes are responsible for promoting cleavage and polyadenylation. CPSF (cleavage and polyadenylation specificity factor) recognizes PAS (polyadenylation signal) and CSTF (cleavage stimulation factor) interacts with DSE (downstream sequence element). Once both of these factors are anchored to the mRNA, an endonuclease in CPSF (polyadenylation speicificity factor) cleaves the messenger between the two sequence elements. The CPSF then recruits PAP (poly(A) polymerase) to extend a tail of uncovered adenosines to the end of the mRNA. The growing tail is coated with nuclear poly (A) binding protein (PABPN). After completing processing, the mature transcripts are exported to the cytoplasm, where PABPN is replaced with its cytoplasmic counterpart, PABP. During translation, PABP interacts with the cap-bound initiation factor eIF4G, improving translation efficiency and ribosome recycling by promoting messenger circularization.

The Clothes Make the mRNA: Past and Present Trends in mRNP Fashion.
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Predicting effective microRNA target sites in mammalian mRNAs.
Elife. 2015 Aug 12;4:e05005. doi: 10.7554/eLife.05005.
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Grimson A, Srivastava M, Fahey B, Woodcroft BJ, Chiang HR, King N, Degnan BM, Rokhsar DS, Bartel DP.
Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals.
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Kim D, Kim J, Baek D.
Global and local competition between exogenously introduced microRNAs and endogenously expressed microRNAs.
Mol Cells. 2014 May;37(5):412-7. doi: 10.14348/molcells.2014.0100. Epub 2014 May 13.
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Taliaferro JM, Lambert NJ, Sudmant PH, Dominguez D, Merkin JJ, Alexis MS, Bazile C, Burge CB. RNA Sequence Context Effects Measured In Vitro Predict In Vivo Protein Binding and Regulation.
Mol Cell. 2016 Oct 20;64(2):294-306. doi: 10.1016/j.molcel.2016.08.035. Epub 2016 Oct 6.
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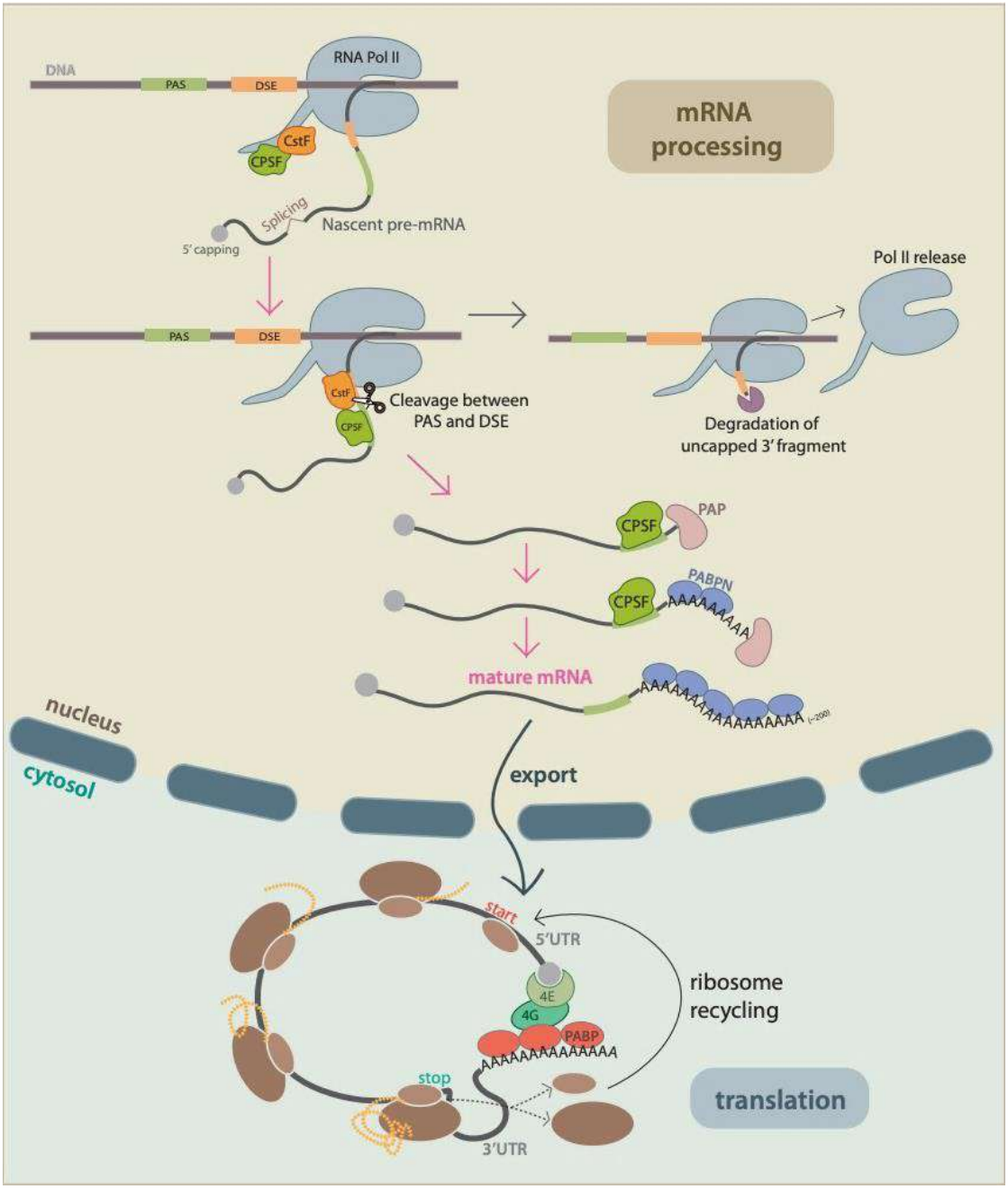
Zlotorynski, E.
The short tail that wags the mRNA.
Nat Rev Mol Cell Biol 19, 2-3 (2018). https://doi.org/10.1038/nrm.2017.120
https://www.nature.com/articles/nrm.2017.120



According to previous studies, long poly-A sequences were preferable for mRNA stability.
Poly-A sequences of 120 units provide more stable IVT-mRNAs and more efficient translation than shorter tails in DCs derived from human monocytes[56] , and in primary human T cells a poly-A tail longer than 300 nucleotides is more favorable for efficient translation. [57]

These results are not inconsistent with the pruning model because IVT-mRNAs with medium and long poly-A tails can initially recruit PABP and be shortened to a 30-A tail.

[56] Holtkamp S, Kreiter S, Selmi A, Simon P, Koslowski M, Huber C, Türeci O, Sahin U.
Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells.
Blood. 2006 Dec 15;108(13):4009-17. doi: 10.1182/blood-2006-04-015024. Epub 2006 Aug 29.
https://ashpublications.org/blood/article/108/13/4009/6595/Modification-of-antigen-encoding-RNA-increases

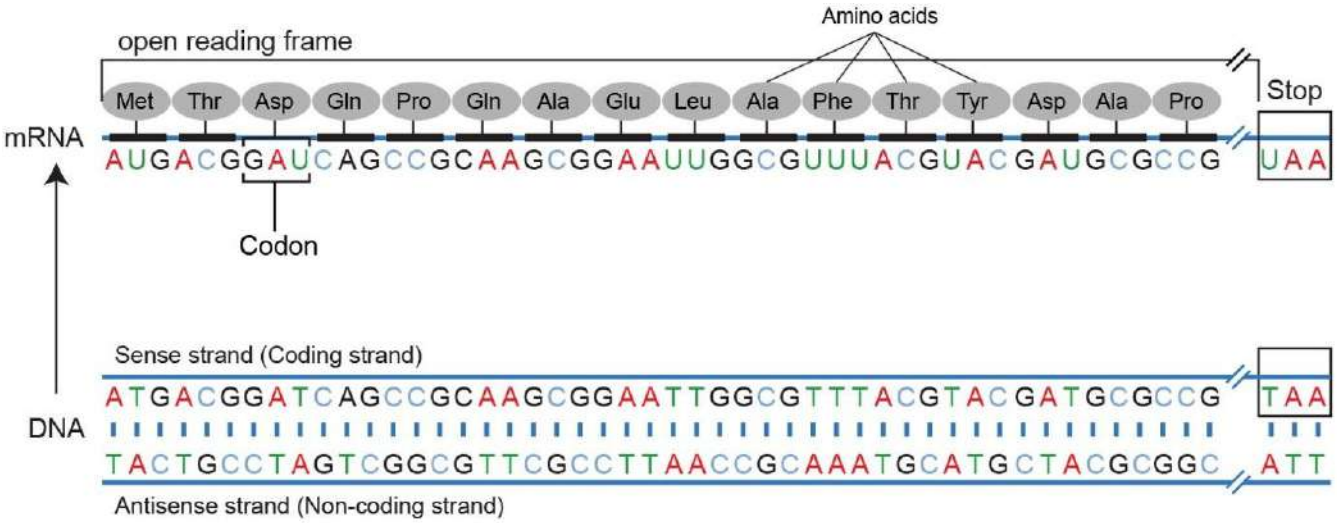
[57] Grier AE, Burleigh S, Sahni J, et al.
pEVL: A Linear Plasmid for Generating mRNA IVT Templates With Extended Encoded Poly(A) Sequences.
Mol Ther Nucleic Acids. 2016;5(4):e306. Published 2016 Apr 19. doi:10.1038/mtna.2016.21
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5014522/

Further studies should evaluate the role of poly-A size for IVT-mRNA antigen expression over time, as ideally, antigen expression should be achieved once APCs migrate to the draining lymph node.

Translated regions

Several approaches have been performed to modify the ORF (open reading frame) sequence* to improve transcriptional efficiency and to prevent a strong innate immune reaction due to PRR recognition.

** Codons with specific functions*
- ***Onset codon (AUG)**: signals the initiation of polypeptide chains in all cells; encodes for the AA METIONIN.*
- ***Termination codons**: there are three (UAA, UAG, UGA) and they do not code for any AA. They signal the end of the polypeptide chain.*
- ***Open** Reading Frame **(ORF)** also referred to as Open Reading Frame. It occurs if a frame does not present a termination codon for more than 50 consecutive nucleotides.*
- ***Codon degeneration**: this is perhaps the most striking feature of the genetic code. Each individual AA can correspond to more than one codon. The difference is usually realized at the third nucleotide.*



The ORF can be modified at the codon level (codon usage bias) to regulate the rate of translation elongation or via GC content to avoid secondary structures, and codon optimization to improve translation and mRNA stability has been evaluated. [58]

There are several strategies for codon optimization; for example, using the most frequent codons for each amino acid, or even using codons with higher tRNA abundance. [59]

[58] Hanson G, Coller J.
Codon optimality, bias and usage in translation and mRNA decay.
Nat Rev Mol Cell Biol. 2018;19(1):20-30. doi:10.1038/nrm.2017.91
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6594389/

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BioDrugs. 2018 Feb;32(1):69-81. doi: 10.1007/s40259-018-0261-x.
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A critical analysis of codon optimization in human therapeutics.
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Protein Expr Purif. 2003 Oct;31(2):247-9. doi: 10.1016/s1046-5928(03)00213-4.
https://pubmed.ncbi.nlm.nih.gov/14550643/

Another strategy is to optimize di-codon usage, that is, to use the best codon pairs that are optimal together. [60]

A third strategy is to modify the ORF sequence to have the same ratio of each codon found naturally in the highly expressed proteins of the target species and cells.
Codon optimization increases translation rates[61] and optimal codons near the start codon increase elongation rates[62] , providing high levels of mRNA translation.
In contrast, rare codons provide a low elongation rate that promotes ribosome crowding. This altered elongation allows binding of a DEAD-Box RNA helicase to the transcript and accelerates mRNA decay after 5' decapsidation[63] .

However, rapid extension rates are not always beneficial.
They might prevent adequate folding of the encoded protein, as shown in a luciferase mRNA optimized for the codon that lost 50 percent of its activity[64] . Less frequent codons could provide a lower translation rate and thus adequate protein folding, which is important for achieving proper antigenic conformation. Therefore, depending on the antigen, different codon strategies may be used.

Optimization of all codons may be of interest in the case of mRNA vaccines based on linear epitopes.
In contrast, complex antigens may require slower translation rates to fold critical protein domains. In any case, the use of rare codons should be avoided in both strategies to optimize protein expression. [65]
In addition, GC content may modify protein expression by influencing translation depending on the stage of cell differentiation;[66] it follows that GC content in mRNA vaccines could be important for target immune cells depending on cellular status (e.g., monocytes vs. macrophages).
Uridine depletion, which may be related to increased GC content, showed improved

[60] Alexaki A, et al
Codon and Codon-Pair Usage Tables (CoCoPUTs): Facilitating Genetic Variation Analyses and Recombinant Gene Design.
J Mol Biol. 2019 Jun 14;431(13):2434-2441. doi: 10.1016/j.jmb.2019.04.021. Epub 2019 Apr 26.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4889334/

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Translation elongation can control translation initiation on eukaryotic mRNAs.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6555954/

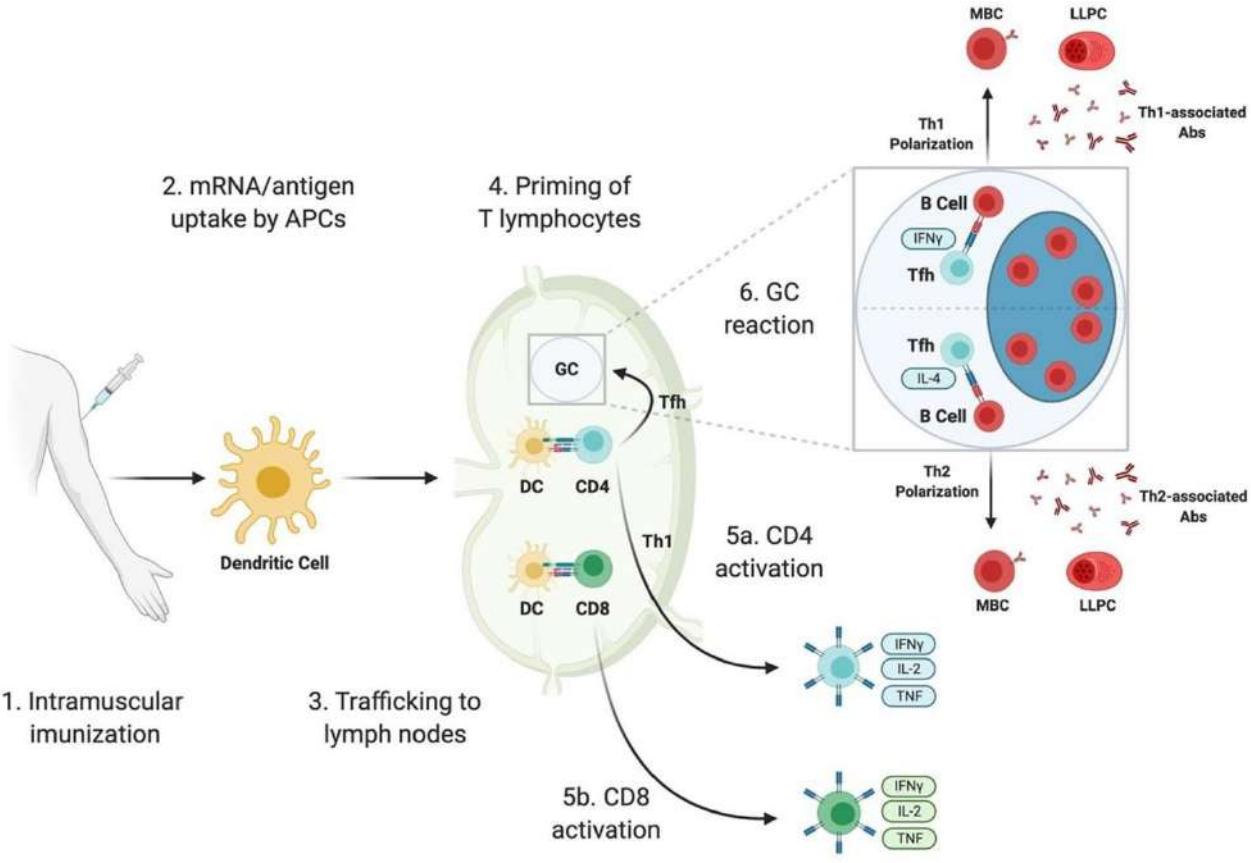
of protein expression in IVT-mRNA, as uridine-rich regions are recognized by RIG-I and its activation blocks the translation mechanism. [67]

Finally, mRNA secondary structures can play an important role in mRNA translation. For example, highly stable secondary structures and hairpin loops should be avoided.
These structures can prevent ribosome entry, scanning, and elongation and be recognized as PAMPs by the innate immune system.

OPTIMIZATION OF IVT -m RNA PRODUCTION FOR STIMULATION OF INNATE IMMUNITARY RESPONSE.

For an injectable mRNA vaccine, the main efficacy considerations include the following: [68]

1) the level of antigen expression in professional antigen-presenting cells (APCs), which is influenced by the efficiency of the vector, the presence of pathogen-associated molecular patterns (PAMPs) in the form of double-stranded RNA (dsRNA) or unmodified nucleosides, and the level of RNA sequence optimization (codon usage, G:C content, 5' and 3' untranslated regions (UTRs) ect);
2) maturation and migration of dendritic cells (DCs) to secondary lymphoid tissue, favored by PAMPs;
3) the vaccine's ability to activate robust follicular T helper cell ($_{TFH}$) and germinal center (GC) responses, an area that remains poorly understood.



https://www.mdpi.com/2076-393X/9/2/147/htm

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Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification.
Mol Ther Nucleic Acids. 2018;12:530-542. doi:10.1016/j.omtn.2018.06.010
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6076213/

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SARS-CoV-2 mRNA Vaccines: Immunological Mechanism and Beyond.
Vaccines. 2021; 9(2):147. https://doi.org/10.3390/vaccines9020147
https://www.mdpi.com/2076-393X/9/2/147/htm

Immune responses elicited by SARS-CoV-2 mRNA vaccines. SARS-CoV-2 mRNA vaccines are administered intramuscularly (1). Either mRNA-LNP or locally produced antigen are taken up by antigen-presenting cells (APCs) (2), such as dendritic cells (DCs). These APCs then transfer to the lymph nodes (3) where they are able to trigger CD4 and CD8 T lymphocytes (4). The events in (2) - (4) are reviewed in detail in [36]. The priming of CD8 T cells can induce the formation of cytotoxic T lymphocytes (5b) capable of directly killing infected cells. Antigen primed CD4 T cells can differentiate into Th1 cells (5a) or follicular helper T cells (Tfh). Tfh cells help initiate a germinal center (GC) reaction (6). Vaccination-induced GC reactions will result in the formation of affinity-matured memory B cells (MBCs) and long-lived plasma cells (LLPCs) that secrete antibodies. Tfh cells can be tilted toward a Th1 or Th2 phenotype, which will influence the class transition of antibodies (Abs) produced b y LLPCs to Th1- or Th2-associated Abs (6).

Insight The germinal center (GC) and follicular T helper cells ($_{TFH}$)

Most potent antimicrobial vaccines induce long-lasting antibody responses against the target pathogen.
High-affinity antibodies are produced at specialized microanatomical sites within B-cell follicles present in secondary lymphoid organs called germinal centers (*GCs*).
B-cell proliferation, somatic hypermutation, and high-affinity antibody selection occur in GCs, and efficient T-cell help is needed for these processes. [69]

$_{TFH}$ cells represent a specialized subset of CD4 T cells^{+} that produce signals critical for B-cell survival, proliferation, and differentiation as well as signals for isotype switching of antibodies and for introducing diversifying mutations in immunoglobulin genes. [70]

$_{TFH}$ cells are predominantly resident in the lymph nodes (LNs) and spleen (except in pathological situations as will be seen below in the interaction between microbiota and $_{TFH}$ cells) because their primary purpose is to help B cells, whereas $_{non\text{-}TFH}$ effector cells such as Th1, Th2, Th17, cytotoxic CD4 T cells, or Th9 cells are predominantly intended to leave lymphoid tissue and move to sites of infection or inflammation, as actions at these sites are the primary purpose of most $_{non\text{-}TFH}$ cells.
The help provided by $_{GC\text{-}TFH\ cells\ to}$ *GC-B* cells is mediated by CXCL13, in addition to more widely expressed cytokines such as IL-2 and TNF. The combined expression of IL-21, IL-4 and CD40L by $_{GC\text{-}TFH\ cells}$ efficiently supports *GC-B* cells. [71]

The interactions between TFHs and GC-B cells resemble cellular connections and synaptic communication within the nervous system, which allow signals to be transduced quickly and efficiently through the synaptic cleft.
Such "immunological synapses" are particularly critical in the *GC* where the speed of T-B cell interactions is greater and their duration is shorter than at other sites.
In the context of *GCs*, which contain large numbers of cells in a highly compact structure, the targeted release of signals between interacting cells becomes especially important.
he promiscuous or third-party release of positive selection signals could potentially lead to the emergence of long-lived self-reactive B-cell clones.

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T cell interactions with B cells during germinal center formation, a three-step model.
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T Follicular Helper Cell Biology: A Decade of Discovery and Diseases.
Immunity. 2019 May 21;50(5):1132-1148. doi: 10.1016/j.immuni.2019.04.011.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6532429/

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T follicular helper cell differentiation, function, and roles in disease.
Immunity. 2014 Oct 16;41(4):529-42. doi: 10.1016/j.immuni.2014.10.004.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4223692/

Weinstein JS, Herman EI, Lainez B, et al.
$_{TFH}$ cells progressively differentiate to regulate the germinal center response.
Nat Immunol. 2016;17(10):1197-1205. doi:10.1038/ni.3554
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5030190/

Cytokines, cytotoxic granules, and more recently neurotransmitters have been shown to be transferred from TFHs to B cells as a result of specific interactions.[72]

TFH cells in infectious and autoimmune diseases and vaccinations

The main function of TFH cells is to provide protection from infectious diseases, as they facilitate antibody responses to viral, bacterial, parasitic, and fungal infections.
For example, the IgG response to vaccine virus infection is reduced by 98% in the absence of TFH cells.[73]

Data from infection studies indicate a primary role of TFH cells in limiting the development of autoreactive B cells.
Although TFH cells are rare early (day 10) in influenza or LCMV infection, they accumulate substantially over time (day 30) and prevent the development of autoreactive (anti-histone) B cells[74] .
This regulatory action is of great significance in preventing or, in the case of dysregulation, inducing autoimmune diseases.[75]

An exaggerated expansion of TFH cells results in an overreaction of the germinal center, with proliferation of self-reactive B cells, an excess in the differentiation of long-lived plasma cells, as well as an overproduction of high-affinity pathogenic autoantibodies.
The pathological abundance of TFH cells could provide crucial support for the survival of related self-reactive B lymphocytes and escape from tolerance checkpoints in the germinal center.

TFH cell disruption has been reported in patients with various autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and autoimmune thyroid disease, where TFH cells are present more frequently and show a positive correlation with serum autoantibody titer.[76]

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Synaptic Interactions in Germinal Centers.
Front Immunol. 2018;9:1858. Published 2018 Aug 13. doi:10.3389/fimmu.2018.01858
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6099157/

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The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells.
Nat Immunol. 2014;15(7):657-666. doi:10.1038/ni.2912
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4289613/

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Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection.
Nat Immunol. 2017;18(11):1249-1260. doi:10.1038/ni.3837
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5679073/

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Spatial and functional heterogeneity of follicular helper T cells in autoimmunity.
Curr Opin Immunol. 2019 Dec;61:1-9. doi: 10.1016/j.coi.2019.06.005. Epub 2019 Jul 30.
https://pubmed.ncbi.nlm.nih.gov/31374450/

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Functional differentiation and regulation of follicular T helper cells in inflammation and autoimmunity.
Immunology. 2020 Oct 31. doi: 10.1111/imm.13282. Epub ahead of print.
https://pubmed.ncbi.nlm.nih.gov/33128768/

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Insights into the role of follicular helper T cells in autoimmunity.
Immune Netw. 2014 Feb;14(1):21-9. doi: 10.4110/in.2014.14.1.21. Epub 2014 Feb 21.
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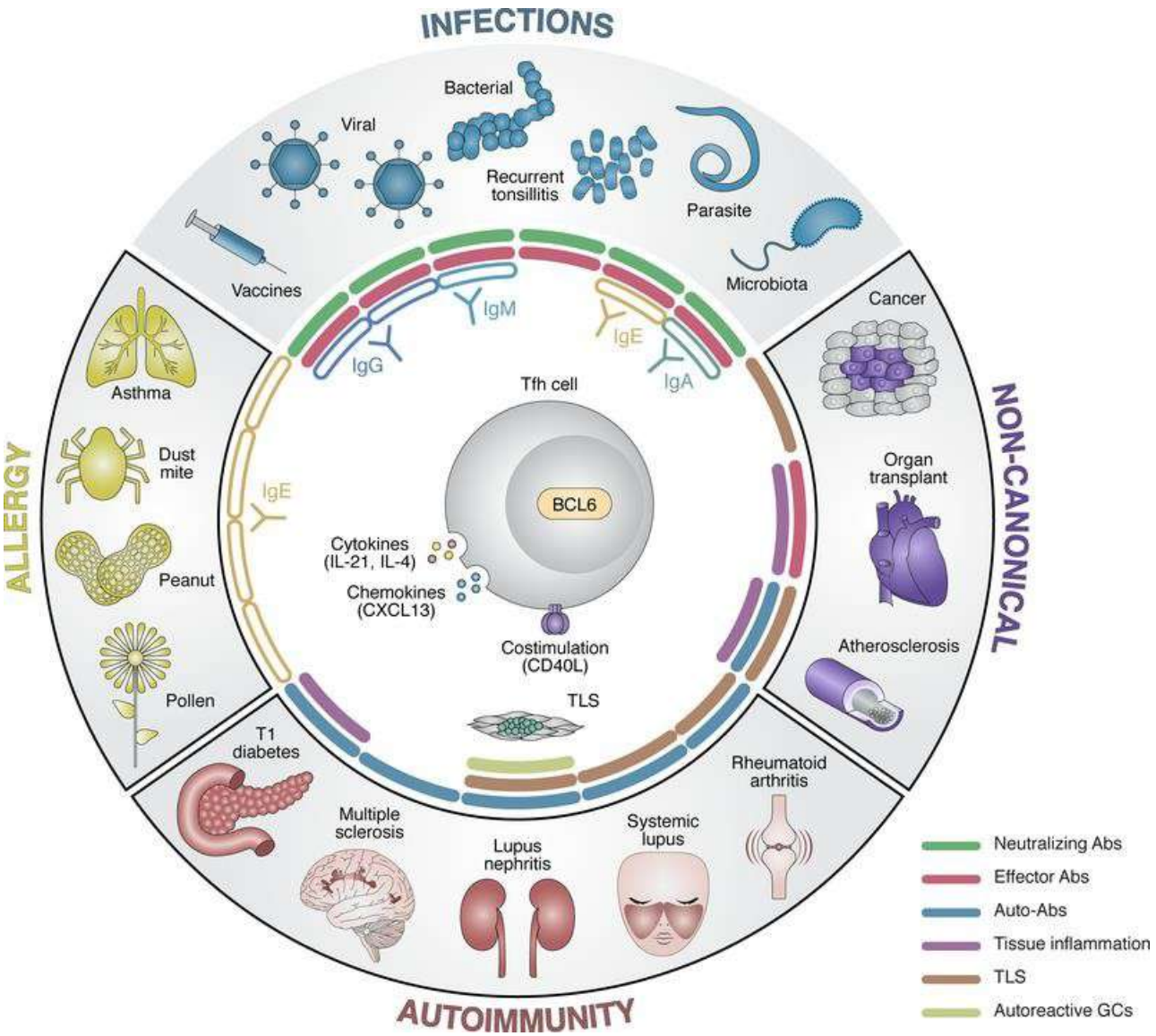
Follicular helper T cells in immunity and autoimmunity.
Braz J Med Biol Res. 2016;49(5):e5209. doi: 10.1590/1414-431X20165209. Epub 2016 Apr 19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4843212/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6532429/

The important role of TFH cells in immune responses to vaccines parallels that in the development of immune responses to pathogen infections; consequently, new adjuvants and vaccine platforms are being developed that can powerfully activate this cell type.

Specifically, it has recently been shown that the m1Ψ-mRNA-LNP vaccine platform induces strong TFH and *GC-B* cell immune responses, which generate high levels of long-lasting neutralizing vaccine antibodies in mice and rhesus macaques.
Two possible actions contributing to this effect have been suggested: the robust and sustained production of antigen from mRNA with modified nucleosides and the adjuvant effects of vaccine LNP promoting *GC-B* cell activation. [77]

Interaction between TFH cells and the microbiota

In addition to facilitating protective immunity against pathogens, TFH cells are important regulators of the microbiota by generating high-affinity IgA responses in Peyer's plaques, follicles found in the associated lymphoid tissue

[77] Pardi N, Hogan MJ, Naradikian MS, et al.
Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses.
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https://pubmed.ncbi.nlm.nih.gov/29739835/

to the gut (GALT)[78] , rich in *GC* against pathogens[79] , and vice versa the gut microbiota affects the cell biology of $_{TFH}$ [80].

Segmented filamentous bacteria ([SFB] Candidatus arthromitus) belong to the most aggressive class of microorganisms found within the microbiota.
This organism evolved in a deep niche attached to the epithelial cells of the lower small intestine.
The location is ideal for SFBs to collect the many amino acids they need from the host or diet (their genome has eliminated the necessary synthetic pathways).
They use special structures called "holdfasts" ("sockets") to anchor themselves to the epithelial layer and avoid being excreted into the large intestine where it would be much more difficult to meet essential amino acid requirements.[81]

In addition to eliciting extremely effective IgA responses (which appear to limit overgrowth during colonization), SFBs induce a remarkably specific and large population of mucosal TH17 cells, which although not directly proinflammatory, under the right susceptibility conditions can provide the germinal center needed to generate arthritis via autoantibodies.

An urgent question being sought to be answered is the mechanism by which the gut microbiota predisposes its host to disease at sites distal from the gut.

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Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense.
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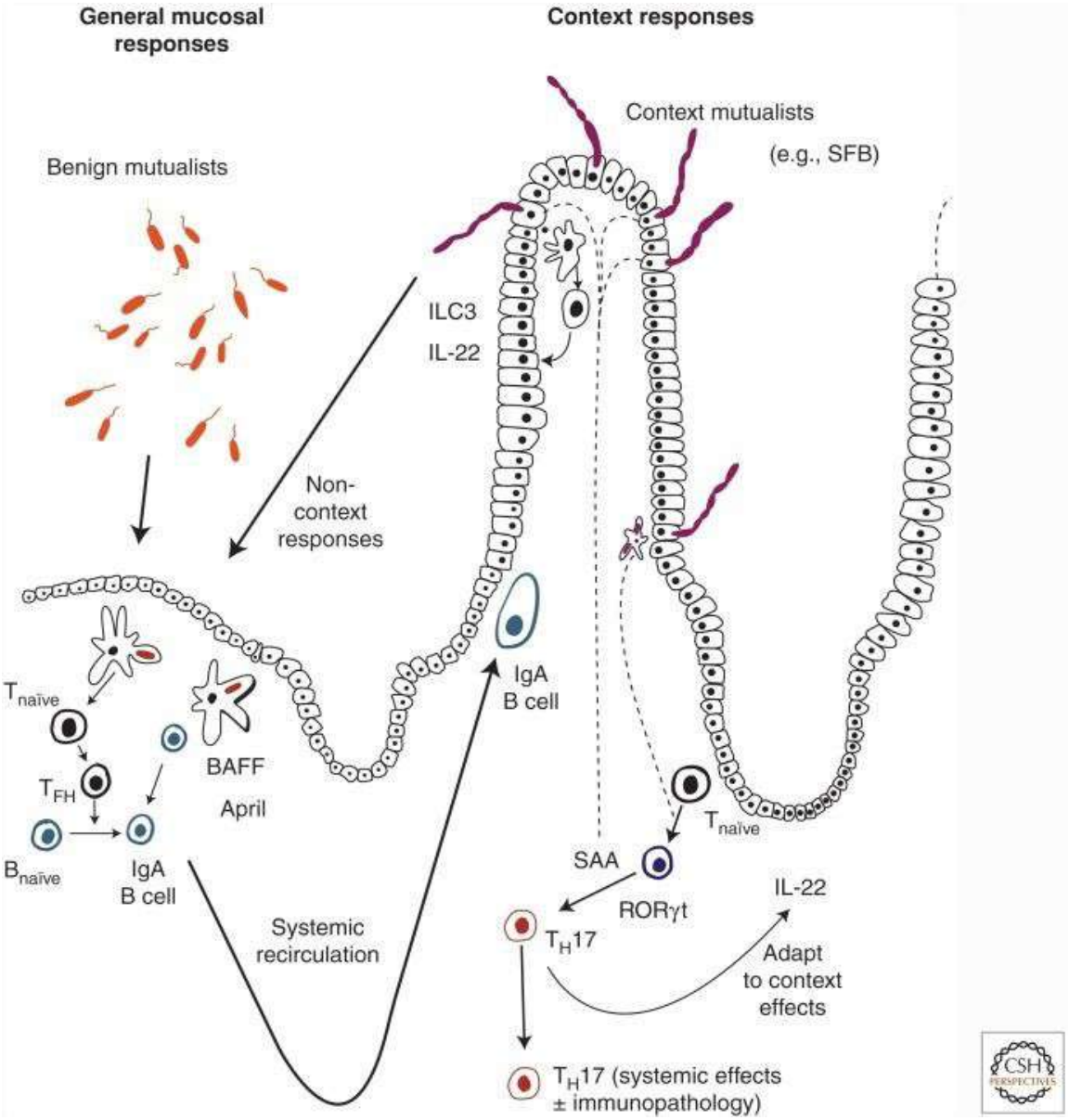
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5793759/

Preite S, Cannons JL, Radtke AJ, et al.
Hyperactivated PI3Kδ promotes self and commensal reactivity at the expense of optimal humoral immunity.
Nat Immunol. 2018;19(9):986-1000. doi:10.1038/s41590-018-0182-3
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6140795/

81 Kuwahara T, et al
The lifestyle of the segmented filamentous bacterium: a non-culturable gut-associated immunostimulating microbe inferred by whole-genome sequencing.
DNA Res. 2011 Aug;18(4):291-303. doi: 10.1093/dnares/dsr022. Epub 2011 Jul 26.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3158464/

Sczesnak A, Segata N, Qin X, Gevers D, Petrosino JF, Huttenhower C, Littman DR, Ivanov II.
The genome of th17 cell-induced segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment.
Cell Host Microbe. 2011 Sep 15;10(3):260-72. doi: 10.1016/j.chom.2011.08.005.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3209701/

Fei et al addressed this question using a model of autoimmune arthritis to clarify how autoimmune signals generated in the gut by intestinal commensals are transposed to systemic sites. [82]
Their results showed that SFBs increased the $_{TFH}$ cell population not only in Peyer's plaques but also in systemic sites such as the spleen and draining popliteal lymph nodes (PLNs).
SFB-induced $_{TFH}$ cell responses preceded the development of arthritis, and $_{TFH}$ cells were required for SFB-mediated enhancement of autoimmune arthritis, which increased the systemic $_{TFH}$ cell population by driving the differentiation and egress of $_{TFH}$ cells from PPs to systemic sites.
This process was crucial for the development of arthritis because self-antibodies were produced mainly at systemic sites and much less in PPs. The authors demonstrated that SFBs induced $_{TFH}$ cell differentiation in PPs by inhibiting the IL-2 signaling pathway in PPs and identified DCs as the critical cell type required for SFB-mediated $_{TFH}$ cell induction and IL-2 receptor α (IL-2Rα) suppression in PPs.

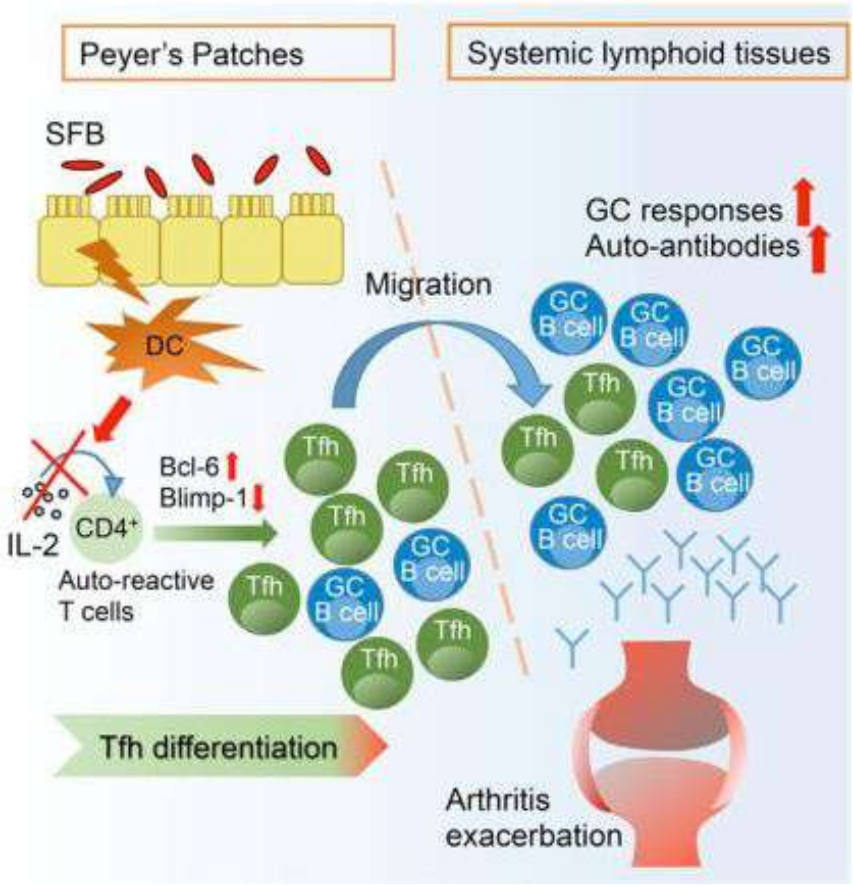


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5793759/
Induction of immune responses by members of the gut microbiota. The extent and character of induction of the mucosal immune response by the microbiota vary depending on the organism involved. Benign noninflammatory mutualists residing in the lumen or confined to the outer mucus layers induce B and T lymphocyte responses within the lymphoid structures of the gut and its draining lymph nodes. These are largely concentrated on the mucosa itself, because the induced lymphocytes mainly return to the gut after systemic recirculation through the lymph vessels and bloodstream. B-cell responses can be induced with the help of T cells or as a result of direct stimulation of B cells by cell-activating factor

[82] Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, Umesaki Y, Wu HJ.
Gut Microbiota Drive Autoimmune Arthritis by Promoting Differentiation and Migration of Peyer's Patch T Follicular Helper Cells. Immunity. 2016 Apr 19;44(4):875-88. doi: 10.1016/j.immuni.2016.03.013.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5296410/

B family of tumor necrosis factor (BAFF) and a proliferation-inducing ligand (APRIL) secreted by mononuclear cells. At the other end of the spectrum, organisms with particularly intimate niches induce a range of additional idiosyncratic immune responses. One example is segmented filamentous bacteria (SFB), which are responsible for generating a substantial population of mucosal-specific TH17 cells, with signals from the serum amyloid protein (SAA) secreted by epithelial cells that bind the microbe in the lower small intestine. SFB also induces specific IgA, which limits overgrowth during colonization. The epithelial layer to which SFB is attached is supported by interleukin (IL)-22 secreted by class 3 innate lymphoid cells (ILC3) through a feedback loop. Although SFB-induced TH17 cells are not inflammatory, under the favorable conditions of major histocompatibility complex (MHC) autoimmune predisposition, they can potentiate B-cell-dependent autoimmune arthritis.

A central aspect of this research is that PP $_{TFH}$ cells could act as the "remote control signal" sent by the gut microbiota to regulate the immune system at distal sites and mediate distal intestinal autoimmune responses; these findings were supported by a recent study that revises the long-standing erroneous assumption that $_{TFH}$ cells are confined to the *GCs* from which they are derived. [83]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5296410/pdf/nihms776862.pdf

There are likely multiple mechanisms by which variation in the gut microbiota can alter the differentiation or function of $_{TFHs}$, and this is an area ripe for future study, given the broad relevance of the microbiota to the physiology of the immune system.

$_{TFH}$ cells in allergies

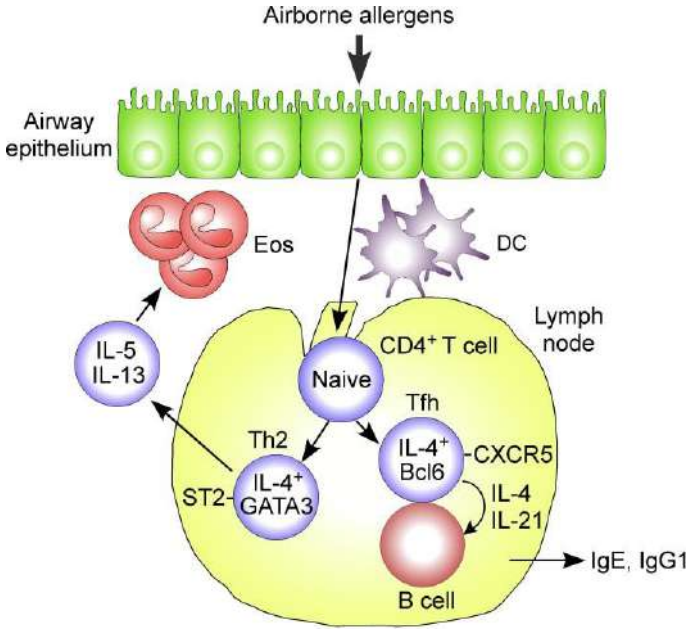
In antibody-mediated allergies, the action of $_{TFH}$ cells is required. It is still commonly assumed that IgE responses are mediated by Th2 cells[84] , but this is incorrect.

[83] Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, Victora GD.
T follicular helper cell dynamics in germinal centers.
Science. 2013 Aug 9;341(6146):673-7. doi: 10.1126/science.1241680. Epub 2013 Jul 25.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3941467/

Kobayashi T, Iijima K, Dent AL, Kita H.
Follicular helper T cells mediate IgE antibody response to airborne allergens.
J Allergy Clin Immunol. 2017;139(1):300-313.e7. doi:10.1016/j.jaci.2016.04.021
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5115999/

[84] Gould HJ, Ramadani F.

It was first demonstrated in 2012, using a combination of experimental approaches in mouse models of helminth infection, that L-4 derived from TFH cells, not IL-4 derived from Th2 cells, was the source for the induction of IgE[85] .
In addition, most of the CD4 T cells⁺ that produce IL-4 in the LNs and spleen are TFH cells, whereas Th2 cells are found in peripheral tissues.[86]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5115999/

In a recent study, Kobayashi et al exposed the airways of naïve mice to cytokines and natural allergens and examined the development of adaptive type 2 immune responses.
A new concept derived from this research is that two major components of allergic immune responses, IgE antibody production and type 2 cytokine responses, were separately regulated by TFH and Th2 cells *in vivo*.
The conventional model suggests that CD4⁺ Th2-type T cells play a key role in various features of allergic immune responses, including IgE antibody production, type 2 cytokine production, airway eosinophilia, and mucosal hyperplasia. [87]
The results of this study suggest that not only were TFH cells necessary for IgE antibody production, but that TFH cells were sufficient even when canonical Th2 cells were absent or their effective functions were impaired.

Peanut allergen-specific antibodies go public.
Science. 2018 Dec 14;362(6420):1247-1248. doi: 10.1126/science.aav3709.
https://science.sciencemag.org/content/362/6420/1247.long

[85] Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM.
Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity.
Nat Immunol. 2011 Dec 4;13(1):58-66. doi: 10.1038/ni.2182.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3242938/

[86] Reinhardt RL, Liang HE, Locksley RM.
Cytokine-secreting follicular T cells shape the antibody repertoire.
Nat Immunol. 2009 Apr;10(4):385-93. doi: 10.1038/ni.1715. Epub 2009 Mar 1.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2714053/

[87] Corry DB, Kheradmand F.
Induction and regulation of the IgE response.
Nature. 1999 Nov 25;402(6760 Suppl):B18-23. doi: 10.1038/35037014.
https://www.nature.com/articles/35037014

Akdis CA.
Therapies for allergic inflammation: refining strategies to induce tolerance.
Nat Med. 2012 May 4;18(5):736-49. doi: 10.1038/nm.2754.
https://www.nature.com/articles/nm.2754

In contrast, Th2 cells had played an important role in type 2 cytokine production and eosinophilic airway inflammation even in the absence of TFH cells or IgE antibodies, which suggested that TFH cells and Th2 cells play distinct roles in allergic immune responses.

Therefore, there is a likelihood that allergic immune responses consist of two distinct subsets of IL-4-producing $CD4^+$ T cells, each of which exhibits unique phenotype, localization, and effector organ functions as summarized in Figure above.

With the extension of this concept, it has been hypothesized that some human allergic diseases may involve TFH cells rather than Th2 cells, and examples might be allergic diseases involving demonstrated roles for IgE antibodies and minimal signs of mucosal inflammation, such as food allergies and anaphylaxis.[88]

The strategies used for the development of mRNA constructs will be discussed in detail below

Generation of an effective IVT-mRNA

As already anticipated, mRNAs used in vaccinology are produced *in vitro* using a phage RNA polymerase (T7, T3 or SP6) and a DNA template.
The types of DNA molds that can be used to produce IVT transcripts are either a PCR product[89] or a linearized plasmid[90] .
During IVT, phage RNA polymerases produce unwanted dsRNA that can activate innate immunity via MDA-5 by blocking mRNA translation.
Therefore, the decrease of dsRNA is important. The dsRNA species can be reduced during IVT production by lowering the concentration of Mg ,[2+91] using modified nucleosides[92] or by a thermostable T7 RNA polymerase producing mRNA at 50°C, or during purification steps.

The different steps and strategies that lead to the production of an IVT-RNA are summarized in the Figure below.

https://www.cell.com/trends/molecular-medicine/fulltext/S1471-4914(19)30244-8
In vitro transcribed mRNA production and formulation (IVT)
(A) The antigen cDNA sequence encoding for the target pathogen antigen is used to process a transcription vector or PCR fragment that includes the complete mRNA information plus an upstream T7 RNA promoter.
(B) linearized plasmid or PCR products are used for IVT-RNA production using the enzyme T7 RNA polymerase in the presence or absence of modified nucleosides. Capping of mRNA is performed during the transcription step in the presence of CAP analogs (ARCA, Clean-Cap) or in two steps after IVT-mRNA production by enzymatic capping reaction.
(C) Then, reaction products containing the DNA mold, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) capped and uncapped are purified. During purification, the DNA mold is removed by DNase treatment, the 5'-ppp of uncapped RNA is dephosphorylated

[88] Kobayashi T, Iijima K, Dent AL, Kita H.
Follicular helper T cells mediate IgE antibody response to airborne allergens.
J Allergy Clin Immunol. 2017;139(1):300-313.e7. doi:10.1016/j.jaci.2016.04.021
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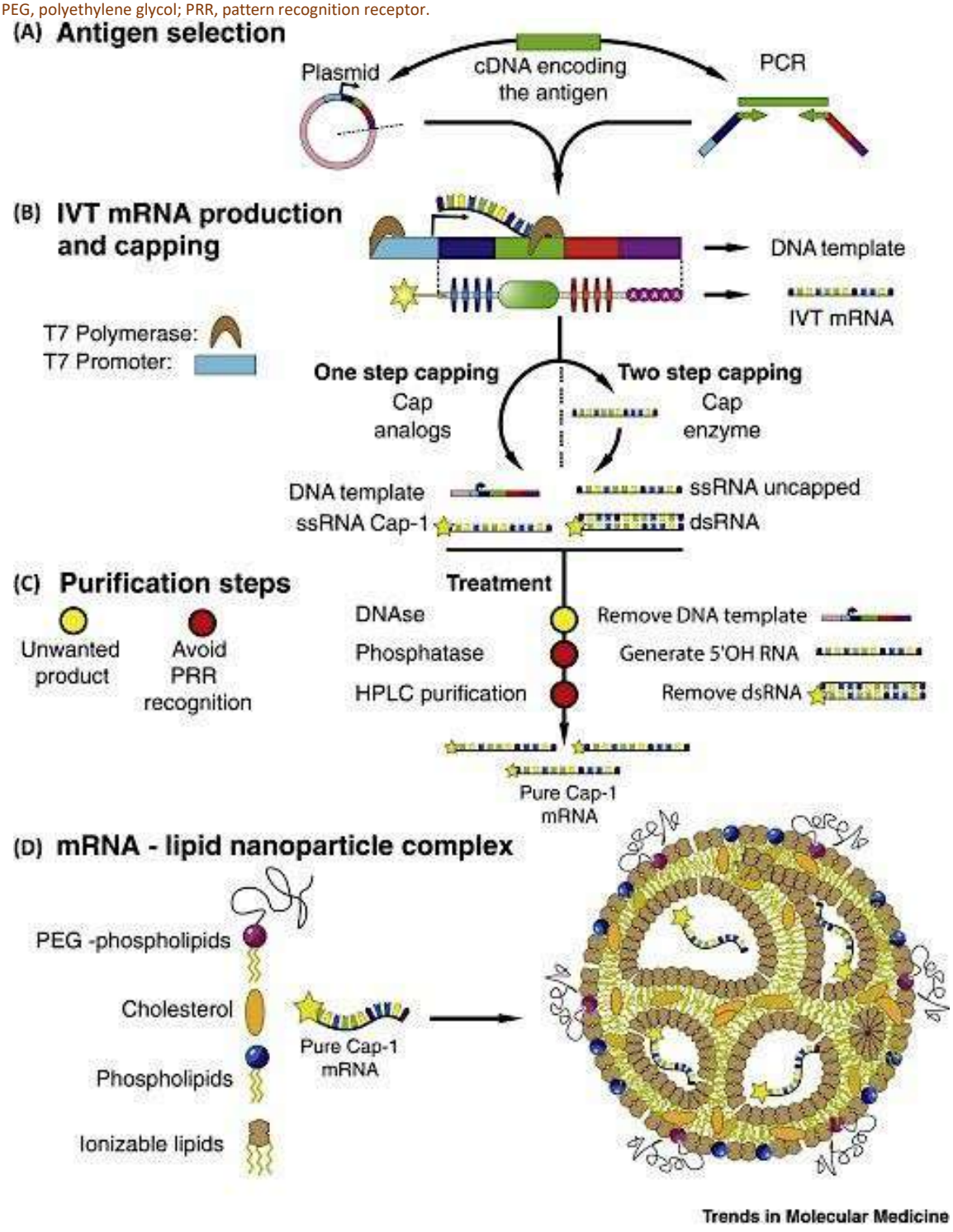
Design, Assembly, Production, and Transfection of Synthetic Modified mRNA.
Methods. 2018 Jan 15;133:29-43. doi: 10.1016/j.ymeth.2017.10.008. Epub 2017 Nov 7.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5807177/

[90] Grier AE, Burleigh S, Sahni J, Clough CA, Cardot V, Choe DC, Krutein MC, Rawlings DJ, Jensen MC, Scharenberg AM, Jacoby K.
pEVL: A Linear Plasmid for Generating mRNA IVT Templates With Extended Encoded Poly(A) Sequences.
Mol Ther Nucleic Acids. 2016 Apr 19;5(4):e306. doi: 10.1038/mtna.2016.21.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5014522/

[91] Mu X, Greenwald E, Ahmad S, Hur S.
An origin of the immunogenicity of in vitro transcribed RNA.
Nucleic Acids Res. 2018 Jun 1;46(10):5239-5249. doi: 10.1093/nar/gky177.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6007322/

[92] Baiersdörfer M, Boros G, Muramatsu H, Mahiny A, Vlatkovic I, Sahin U, Karikó K. A
Facile Method for the Removal of dsRNA Contaminant from In Vitro-Transcribed mRNA.
Mol Ther Nucleic Acids. 2019 Apr 15;15:26-35. doi: 10.1016/j.omtn.2019.02.018. Epub 2019 Feb 27.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6444222/

in 5'OH RNA (if necessary), the dsRNAs are discarded by HPLC or purification on cellulose to obtain a pure, homogeneous solution of the mRNA of interest.
(D) The last step is the formation of an mRNA nanocomplex for efficient cellular transport and transcript translation. For example, we constructed a hypothetical lipid nanoparticle model in which mRNA is trapped in internal droplets in the core of a lipid nanoparticle, providing both protection from RNases and efficient transport to the cytosol.
Abbreviation: PEG, polyethylene glycol; PRR, pattern recognition receptor.



Types of modified nucleosides

Using a cellular mRNA that codes for the protein of interest provides immediate translation of the antigen, but a large dose of synthetic mRNA is required.
To overcome this problem, several approaches have been developed based on a deeper understanding of cell biology.

RNA consists of four nucleotides-guanine, uracil, adenosine, and cytosine-and is designed to have a short half-life in vivo, serving as a carrier of information between DNA and proteins. One challenge in using RNA as a therapy or vaccine for prophylactic purposes has been to control its half-life to ensure therapeutic or vaccine efficacy.
Several methods have been developed to achieve this goal including the use of saRNA, already discussed[93] , and nucleoside modification. [94]

Modified nucleosides can increase the potency of the[95] mRNA vaccine in two different ways.
First, they may decrease the amount of dsRNA species generated during the IVT reaction, or impair the recognition and/or activation of PRR. [96]

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Self-assembled mRNA vaccines
[published online ahead of print, 2021 Jan 2]. Adv Drug Deliv Rev. 2021;170:83-112. doi:10.1016/j.addr.2020.12.014
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7837307/

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https://pubmed.ncbi.nlm.nih.gov/27987145/

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Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses.
J Exp Med. 2018;215(6):1571-1588. doi:10.1084/jem.20171450
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5987916/

Willis E, Pardi N, Parkhouse K, Mui BL, Tam YK, Weissman D, Hensley SE.
Nucleoside-modified mRNA vaccination partially overcomes maternal antibody inhibition of de novo immune responses in mice.
Sci Transl Med. 2020 Jan 8;12(525):eaav5701. doi: 10.1126/scitranslmed.aav5701.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7339908/

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Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses.
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Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability.
Mol Ther. 2008 Nov;16(11):1833-40. doi: 10.1038/mt.2008.200. Epub 2008 Sep 16.
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Vaidyanathan S, et al
Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification.
Mol Ther Nucleic Acids. 2018 Sep 7;12:530-542. doi: 10.1016/j.omtn.2018.06.010. Epub 2018 Jun 30.
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Modified mRNA Vaccines Protect against Zika Virus Infection.
Cell. 2017 Mar 9;168(6):1114-1125.e10. doi: 10.1016/j.cell.2017.02.017. Epub 2017 Feb 17. Erratum in: Cell. 2017 Mar 23;169(1):176.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5388441/

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RNAs Containing Modified Nucleotides Fail To Trigger RIG-I Conformational Changes for Innate Immune Signaling.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5030355/

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Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA.
Immunity. 2005 Aug;23(2):165-75. doi: 10.1016/j.immuni.2005.06.008.
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Charette M, Gray MW.
Pseudouridine in RNA: what, where, how, and why.
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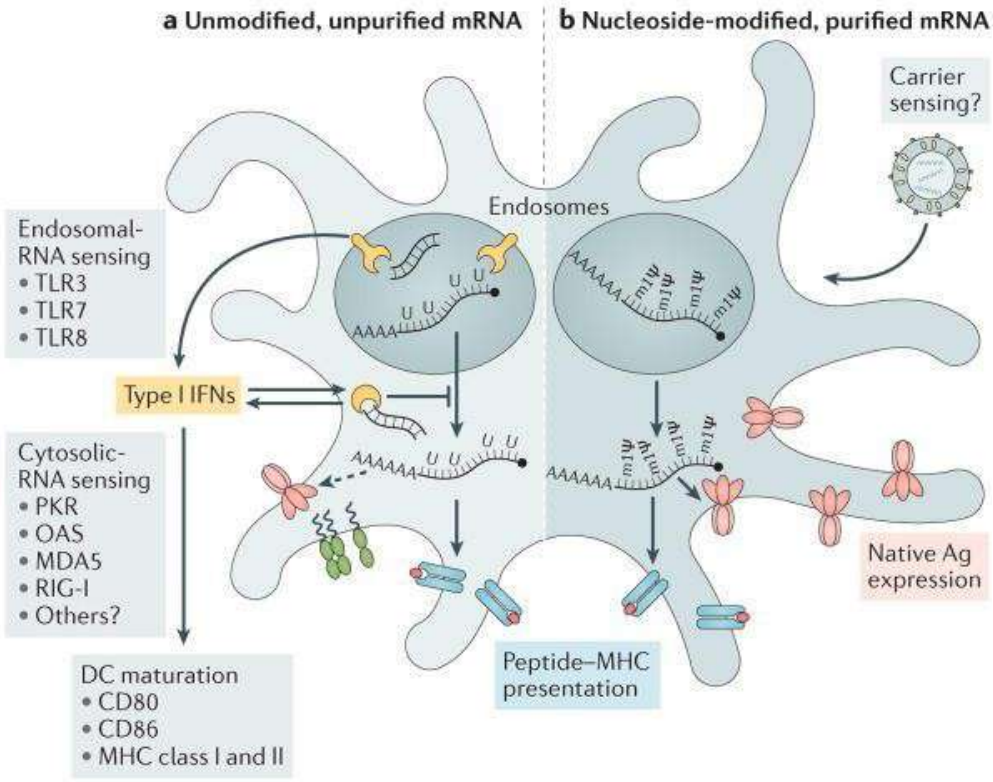
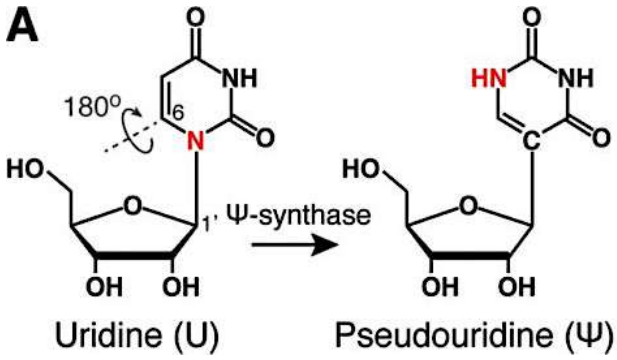
Freund I, Eigenbrod T, Helm M, Dalpke AH.
RNA Modifications Modulate Activation of Innate Toll-Like Receptors.
Genes (Basel). 2019 Jan 29;10(2):92. doi: 10.3390/genes10020092.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5906799/

Innate immune detection by part of mRNA vaccines
Innate immune detection of two types of mRNA vaccines by a dendritic cell (DC), with the sensors for RNA shown in yellow, antigen in red, DC maturation factors in green, and peptide-MHC complexes in blue and red; an example of a lipid nanoparticle vector is shown in the upper right. A non-exhaustive list of known major RNA sensors contributing to the recognition of single-stranded, double-stranded and unmodified mRNAs is shown. Unmodified, unpurified (part a) and nucleoside-modified, and purified rapid protein liquid chromatography (FPLC) (part b) mRNAs were selected for illustration of two mRNA vaccine formats in which known forms of mRNA sensing are present and absent, respectively. The dashed arrow represents the expression reduced antigen. Ag, antigen; PKR, interferon-induced double-stranded RNA-activated protein kinase; MDA5, interferon-induced helicase C domain-containing protein 1 (also known as IFIH1); IFN, interferon; m1Ψ, 1-methylpseudouridine; OAS, 2-5′-oligoadenylate synthetase; TLR, Toll-like receptor.

One modification that has been shown to increase the biological stability of mRNA is the replacement of uridine with pseudo-uridine (Ψ).[97]



[97] Karikó K, Muramatsu H, Welsh FA, Ludwig J, Kato H, Akira S, Weissman D.
Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability.
Mol Ther. 2008 Nov;16(11):1833-40. doi: 10.1038/mt.2008.200. Epub 2008 Sep 16.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2775451/

Charette M, Gray MW.
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IUBMB Life. 2000 May;49(5):341-51. doi: 10.1080/152165400410182.
https://iubmb.onlinelibrary.wiley.com/doi/epdf/10.1080/152165400410182

Spenkuch F, Motorin Y, Helm M.
Pseudouridine: still mysterious, but never a fake (uridine)!!!
RNA Biol. 2014;11(12):1540-1554. doi:10.4161/15476286.2014.992278
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4615568/

Yu YT, Meier UT.
RNA-guided isomerization of uridine to pseudouridine--pseudouridylation.
RNA Biol. 2014;11(12):1483-1494. doi:10.4161/15476286.2014.972855
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4615163/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4615163/

This is a modification that can occur naturally *in vivo,* in which Ψ is derived from uridine (U) via base-specific isomerization catalyzed by Ψ-synthase, and functions to increase the half-life of mRNA (epigenetic RNA modification or epi-transcriptomic).[98]

Mammals do not metabolize Ψ, as they do not produce the enzyme that degrades the glycosidic C-C bond, but eliminate the intact nucleoside by urinary route.[99]
Recently, a pseudouridine-5'-phosphatase has been described that defosphorylates Ψ in humans.[100] Since analyses performed on cell extracts demonstrate the conversion of pseudouridine-5'-phosphate to triphosphate, defosphorylation[101] could prevent the accidental incorporation of pseudouridine into RNA transcripts.
In eubacteria, however, there is a specific degradation pathway for Ψ, in which first it is phosphorylated by a kinase dedicated and subsequently converted to uracil and ribose-5'-phosphate. [102]

The *in vitro* transcription used to generate the mRNA allows this substitution to be easily achieved because pseudo-uridine rather than uridine is added to the transcription reaction. This technology is used to construct the mRNA vaccines for COVID-19 developed by Moderna and Pfizer.

Insight **Nature-modified nucleosides and the evolution of RNA**

Nucleoside modification is the foundation of the oldest "immune" mechanism.
Bacteria naturally methylate selected nucleosides in their genome to distinguish and destroy the unmodified DNA of an invader with restriction enzymes.
During evolution, discrimination between host and pathogen based on DNA methylation characteristics remains an important component of the immune system. [103]

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[98] Li X, Ma S, Yi C.
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https://www.nature.com/articles/201411a0

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The incorporation of 5-ribosyluracil triphosphate into RNA in nuclear extracts of mammalian cells.
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Molecular identification of pseudouridine-metabolizing enzymes.
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Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA.
Immunity. 2005 Aug;23(2):165-75. doi: 10.1016/j.immuni.2005.06.008.
https://www.cell.com/immunity/fulltext/S1074-7613(05)00211-6

Ishii KJ, Akira S.
TLR ignores methylated RNA?
Immunity. 2005 Aug;23(2):111-3. doi: 10.1016/j.immuni.2005.08.003.

Pathogen detection by the innate immune system relies on a small subset of germline-encoded pattern recognition receptors that recognize structurally conserved microbial molecules.
In addition to the cell wall components of bacteria and fungi, nucleic acids (NA) have been identified as powerful stimuli that initiate a robust immune response.
Since both "self" and "non-self" nucleic acids are composed of the same basic building blocks, the ability to identify the origin of DNA and RNA is a crucial necessity for the innate immune system.
In addition to cytosolic receptors, including retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5 (MDA-5), and cyclic GMP-AMP synthase (cGAS), NAs are recognized by Toll -like receptors (TLRs) 3, TLR7, TLR8, TLR9, and TLR13 that reside in the endosome.

Three main mechanisms have been described that allow the innate immune system to avoid recognition of host ("self") NA, but allow stimulation by microbial ("foreign") NA.
First, discrimination may be based on the spatial restriction of NA-sensitive receptors in specific subcellular compartments. Indeed, TLRs reside in the endolysosome to which RNA/DNA-self has no or limited access. In contrast, microbial RNA/DNA may have access after endosomal uptake and degradation of microbes in phagocytes.

Second, nucleotide composition, naturally occurring chemical modifications and distinct sequence motifs influence the primary, secondary and tertiary structures of RNA and DNA and determine NA recognition.
As such, cytosolic 5'-triphosphorylated RNA that is produced during virus RNA replication activates cytosolic RIG-I while eukaryotic "self" RNA avoids recognition through capping. [104]
The unmethylated GC frequency is increased in prokaryotes compared with mammalian DNA and thus induces TLR9 stimulation.

More than 150 different naturally occurring RNA post-transcriptional modifications have been identified[105] .

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https://www.cell.com/immunity/fulltext/S1074-7613(05)00241-4

Freund I, Eigenbrod T, Helm M, Dalpke AH.
RNA Modifications Modulate Activation of Innate Toll-Like Receptors.
Genes (Basel). 2019;10(2):92. Published 2019 Jan 29. doi:10.3390/genes10020092
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6410116/

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Flamme M, McKenzie LK, Sarac I, Hollenstein M.
Chemical methods for the modification of RNA.
Methods. 2019 May 15;161:64-82. doi: 10.1016/j.ymeth.2019.03.018. Epub 2019 Mar 21.
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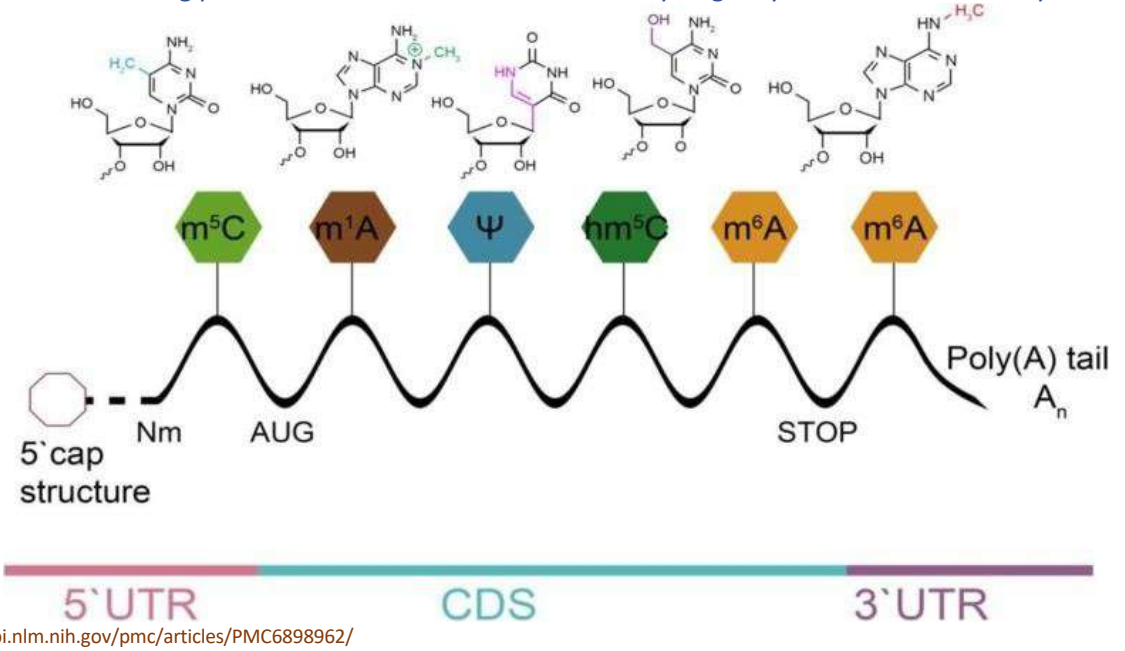
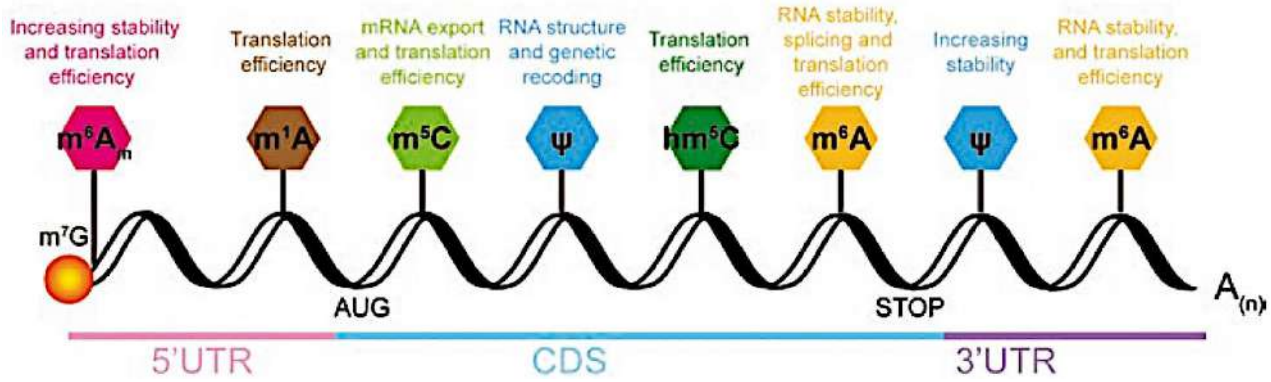
Eukaryotic RNA in general is more heavily modified than prokaryotic RNA, and RNA modifications can be considerably more complex thus allowing potential discrimination of evolutionary origin by the innate immune system.[106]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6898962/



https://www.researchgate.net/publication/334631681_RNA_N_6-Methyladenosine_Modification_in_Normal_and_Malignant_Hematopoiesis

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Dynamic RNA Modifications in Gene Expression Regulation.
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RNA N 6-Methyladenosine Modification in Normal and Malignant Hematopoiesis.
Adv Exp Med Biol. 2019;1143:75-93. doi: 10.1007/978-981-13-7342-8_4.
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[106] Roundtree IA, Evans ME, Pan T, He C.
Dynamic RNA Modifications in Gene Expression Regulation.
Cell. 2017;169(7):1187-1200. doi:10.1016/j.cell.2017.05.045
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Karikó et al. were the first to elucidate differences in immune stimulation by RNA dependent on its evolutionary origin[107] , reporting a negative correlation of the immunostimulatory potential of RNA and the extent of embedded modifications.
Based on the observation that innate cytokine secretion was inefficient with mammalian cytosolic RNA but efficient with bacterial RNA and mitochondrial RNA, the authors specifically analyzed the activation of TLRs3, 7 and 8 by modified RNA.
In vitro transcribed RNA containing randomly incorporated s2U or m6A was unable to stimulate TLR3, while m6A, m5C, m5U, s2U and Ψ modifications prevented stimulation of TLR7 and 8 (see Figure below for structures).

The latter modifications also inhibited TNF and IL-12 secretion from monocyte-derived dendritic cells. However, in primary DC populations the RNA modified by m6A and m5C[108] stimulated TNF secretion, indicating cell type differences.
Recently, the incorporation of N(1)-methylpseudouridine (m1 Ψ) into mRNA, either alone or in combination with m5C, has been shown to further reduce the stimulation of innate immunity, especially through TLR3s compared with pseudo-uridine[109] .

As mRNA stability has been improved in parallel, this modification has been suggested to enhance performance for mRNA-based therapies. Indeed, *in vivo* mRNA vaccines modified with N(1)-methylpseudouridine have been shown to induce improved follicular T helper cell responses and germinal B cell activation, but in fact this may be due to both effects on mRNA stability and reduced innate immune stimulation.[110]

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[107] Karikó K, Buckstein M, Ni H, Weissman D.
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Post-transcriptional pseudouridylation in mRNA as well as in some major types of noncoding RNAs.
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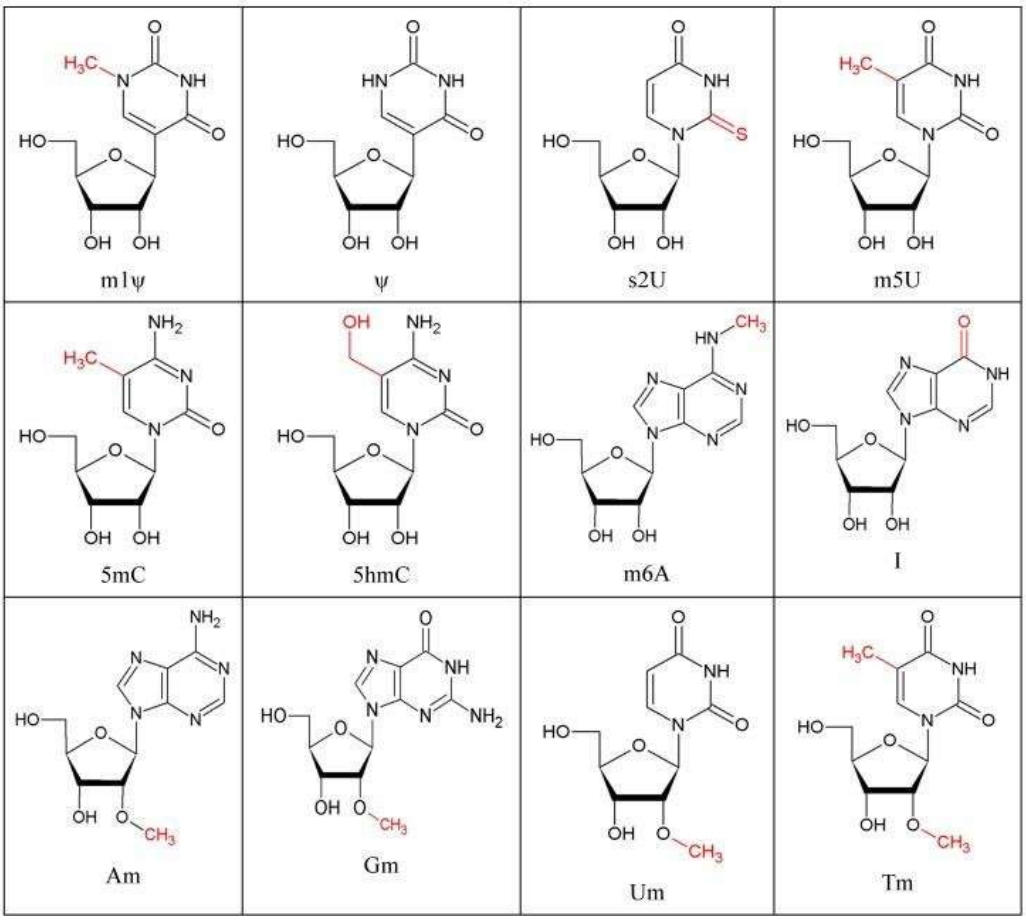
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5906799/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6410116/ Selected RNA modifications with immunomodulatory properties

Of utmost importance, cytosolic detection of RNA-self by RIG-I or MDA-5 is blocked by specific modifications that prevent RNA recognition.

The conversion of A to I by the RNA-acting enzyme adenosine deaminase (ADAR) is an important strategy to make host RNA invisible to the innate immune system.[111]
In fact, adenosine deamination is the most common modification affecting innate control[112] . In short, ADARs hydrolytically deaminate adenosines in the duplex RNA regions, whereby inosine mimics guanosine in hydrogen bonding and RNA function can be significantly modified.
In addition, as already seen, the transcribed RNA in the nucleus is modified with the 5' cap.
This is characterized by an inverted 7-methylguanosine bound to the first transcribed nucleotide (5'-cap) and methylation of the 2'-O-ribose of the first (ch 1) and often the second (ch 2) nucleotide that abolishes interaction with

[111] Liddicoat BJ, Piskol R, Chalk AM, et al.
RNA editing by ADAR1 prevents MDA5 sensing of endogenous dsRNA as nonself.
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A-to-I RNA editing - immune protector and transcriptome diversifier.
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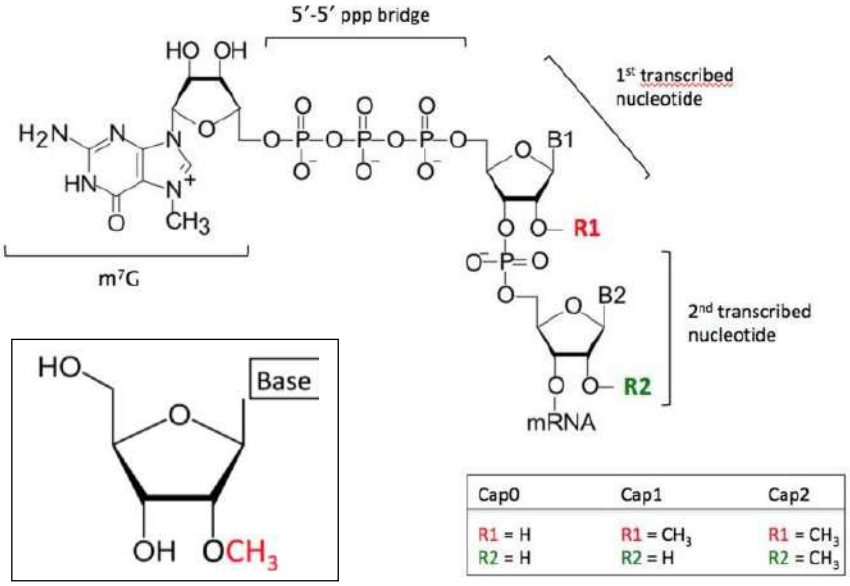
RIG-I and MDA5[113] . In contrast, RNA formed outside the nucleus by viral replication shows a 5'-triphosphate that is recognized by RIG-I.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6409641/
RNA cap structure: the structure of cap0 consists of a guanosine residue, methylated at position N-7, which is linked to the terminal 5′- end nucleotide of mRNA with a 5′-5 ′ triphosphate bridge. Subsequent 2′-O-methylation in the ribose of the first, or both the first and second, nucleotides of transcribed mRNA leads to the formation of cap1 or cap2, respectively.

Thus, through evolutionary processes, viruses such as flaviviruses, coronaviruses and poxviruses have developed 5'-alternative elements including 2'-O-methylation that avoid recognition by the innate immune system.[114]
2'-O-methylation, is also naturally occurring at a significantly higher abundance in eukaryotic RNA than in prokaryotic (and mitochondrial) RNA[115] , suggesting that recognition of "hypomethylated" RNA is used to discriminate RNA-self from foreign RNA.
Methylation of 2'-O ribose is known to act as a dominant inhibitor on TLR7 and TLR8, meaning that methylation of 2'-O of only a proportion of RNA is sufficient to abrogate immune stimulation of the entire RNA preparation.

In addition to pseudouridine, 2'-O-methylation is one of the most abundant post-transcriptional modifications within eukaryotic ribosomal RNA; however, the importance of modifications to regulate immune activation is a double-edged sword that needs to balance adequate immune activation on infection and self tolerance.

[113] Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I.
Science. 2006 Nov 10;314(5801):994-7. doi: 10.1126/science.1132505. Epub 2006 Oct 12.
https://pubmed.ncbi.nlm.nih.gov/17038590/

Schuberth-Wagner C, Ludwig J, Bruder AK, et al.
A Conserved Histidine in the RNA Sensor RIG-I Controls Immune Tolerance to N1-2'O-Methylated Self RNA.
Immunity. 2015;43(1):41-51. doi:10.1016/j.immuni.2015.06.015
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Innate immune restriction and antagonism of viral RNA lacking *2-O-methylation*.
Virology. 2015;479-480:66-74. doi: 10.1016/j.virol.2015.01.019.
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RNA modifications misused by some pathogens can cause immune evasion[116] , while inappropriate processing of self-NA can cause autoimmune diseases.[117]

It is important to keep in mind that in addition to their role in regulating normal cellular processes, RNA modifications also play a role in carcinogenesis and confer stem-like properties to subpopulations of cancer cells.
On the one hand, RNA modifications may contribute to cancer progression by decreasing the stability of oncogene suppressors, thus eliminating their inhibitory effects; on the other hand, they may increase the stability and expression of protooncogene transcripts. [118]

The third mechanism that protects host DNA from self-recognition is nucleic acid degradation by nucleases. One example is cytosolic DNase III, which prevents the accumulation of endogenous DNA and thus the activation of cytosolic DNA sensors.

While under physiological conditions recognition of self-ANs is effectively inhibited, nondiscrimination of self may fail in specific diseases. In fact, it has now been reported that innate immune recognition of host NAs can facilitate the onset and development of autoimmune reactions, for example, in systemic lupus erythematosus and psoriasis. [119]

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[116] Tsai K, Cullen BR.
Epigenetic and epitranscriptomic regulation of viral replication.
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[118] Uddin MB, Wang Z, Yang C.
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RNA. 2020 Dec 29:rna.077271.120. doi: 10.1261/rna.077271.120.
https://rnajournal.cshlp.org/content/early/2020/12/29/rna.077271.120.long

[119] von Landenberg P, Bauer S.
Nucleic acid recognizing Toll-like receptors and autoimmunity.
Curr Opin Immunol. 2007 Dec;19(6):606-10. doi: 10.1016/j.coi.2007.10.004. Epub 2007 Dec 3.
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Heat shock protein 90 associates with Toll-like receptors 7/9 and mediates self-nucleic acid recognition in SLE.
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Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors.
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Recent studies suggest that post-transcriptional epigenomic RNA modifications could be a powerful tool to evade innate immune responses. For example, epigenomic modification by natural acetylation of cytidines increases mRNA translational efficiency. [120]

Overall, studies conducted to optimize transcription in vitro suggest that a decrease in intrinsic innate immune activation of mRNA (such as a decrease in dsRNA contaminants or the use of modified nucleosides) during mRNA production enhances adaptive immune response and mRNA translation.

Post-transcriptional Modification, Capping and Defosphorylation of 5'-mRNA

Two main strategies are used to produce the capped mRNA with IVT.
The first is to use cap analogs such as anti-reverse-cap (ARCA) or Clean Cap[121] in a single reaction. The capping efficiencies are for ARCA 60-80% (CAP-0 structure) and for Clean Cap 90-99% (CAP-1 structure).

The second strategy uses an enzymatic reaction after the mRNA IVT reaction, achieving a higher capping efficiency (100%, CAP-1 structure).
The main advantage of using cap analogs such as ARCA or Clean CAP is a faster, simpler and easier to control process than enzymatic reactions that require an additional capping step.
In addition, to optimize mRNA translation, IVT-mRNAs that remain uncovered should be treated with phosphatase to avoid recognition by the innate immune system, since as already seen RIG-I could recognize the 5' triphosphate uncapped mRNA[122] and block mRNA translation.

[120] Arango D, et al
Acetylation of Cytidine in mRNA Promotes Translation Efficiency.
Cell. 2018 Dec 13;175(7):1872-1886.e24. doi: 10.1016/j.cell.2018.10.030. Epub 2018 Nov 15.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6295233/

[121] Vaidyanathan S, et al
Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification.
Mol Ther Nucleic Acids. 2018 Sep 7;12:530-542. doi: 10.1016/j.omtn.2018.06.010. Epub 2018 Jun 30.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6076213/

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5'-Triphosphate RNA is the ligand for RIG-I.
Science. 2006 Nov 10;314(5801):994-7. doi: 10.1126/science.1132505. Epub 2006 Oct 12.
https://pubmed.ncbi.nlm.nih.gov/17038590/

REVIEW

Strategies for optimizing the pharmacology of mRNA[123]

Numerous technologies are currently being used to improve the pharmacological aspects of mRNA. The various mRNA modifications used and their impact are summarized below.

- Synthetic cap analogs and capping enzymes stabilize mRNA and increase translation of the proteins by binding to eukaryotic translation initiation factor 4E (EIF4E)
- Regulatory elements in the 5'-untranslated region (UTR) and 3'-UTR stabilize mRNA and increase protein translation
- Poly(A) tail stabilizes mRNA and increases protein translation
- Modified nucleosides reduce innate immune activation and increase translation
- Separation and/or purification techniques: treatment with RNase III and purification by rapid protein liquid chromatography (FPLC) reduce immune activation and increase translation
- Sequence and/or codon optimization increases translation
- Modulation of target cells: co-transfer of translation initiation factors and other methods alter translation and immunogenicity

[123] Pardi N, Hogan MJ, Porter FW, Weissman D.
mRNA vaccines - a new era in vaccinology.
Nat Rev Drug Discov. 2018 Apr;17(4):261-279. doi: 10.1038/nrd.2017.243. Epub 2018 Jan 12.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5906799/

PFIZER VACCINE m RNA MODIFICATIONS. [124]

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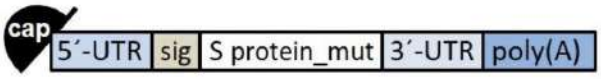
11889

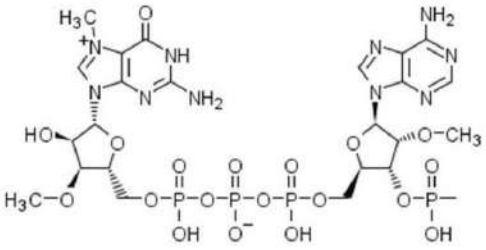
Description

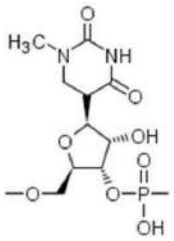
Messenger RNA encoding the full-length SARS-CoV-2 spike glycoprotein.

Schematic



UTR = Untranslated region; sig = extended signal sequence of the S glycoprotein; S protein_mut = S glycoprotein sequence containing mutations K986P and V987P; poly(A) = polyadenylate signal tail.





5′- capping structure
cap G^1A^2 = $m^7G^+m^{3'}$-5'-ppp-5'-$Am^{2'}$-3'-p-
[m^7 = 7-CH_3; $m^{3'}$ = 3'-*O*-CH_3; $m^{2'}$ = 2'-*O*-CH_3;
-ppp- = -PO_2H-O-PO_2H-O-PO_2H)-; -p- = -PO_2H-]

$m^1\Psi$ = 1-methyl-3'-pseudouridylyl

Table of features

Element	Description	Position
cap	A modified 5′-cap1 structure ($m^7G^+m^{3'}$-5'-ppp-5'-Am)	1-2
5′-UTR	5′-untranslated region derived from human alpha-globin RNA with an optimized Kozak sequence	3-54

[124] https://berthub.eu/articles/posts/reverse-engineering-source-code-of-the-biontech-pfizer-vaccine/
https://berthub.eu/articles/posts/italian-reverse-engineering-source-code-of-the-biontech-pfizer-vaccine/

Annette B. Vogel, et al
A prefusion SARS-CoV-2 spike RNA vaccine is highly immunogenic and prevents lung infection in non-human primates
bioRxiv 2020.09.08.280818; doi: https://doi.org/10.1101/2020.09.08.280818
https://www.biorxiv.org/content/10.1101/2020.09.08.280818v1.full.pdf

Orlandini von Niessen AG, Poleganov MA, Rechner C, et al.
Improving mRNA-Based Therapeutic Gene Delivery by Expression-Augmenting 3' UTRs Identified by Cellular Library Screening.
Mol Ther. 2019;27(4):824-836. doi:10.1016/j.ymthe.2018.12.011
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453560/

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International Nonproprietary Names Programme

9/2020

sig	S glycoprotein signal peptide (extended leader sequence), which guides translocation of the nascent polypeptide chain into the endoplasmic reticulum.	55-102
S protein_mut	Codon-optimized sequence encoding full-length SARS-CoV-2 spike (S) glycoprotein containing mutations K986P and V987P to ensure the S glycoprotein remains in an antigenically optimal pre-fusion conformation; stop codons: 3874-3879 (underlined)	103-3879
3'-UTR	The 3´ untranslated region comprises two sequence elements derived from the amino-terminal enhancer of split (AES) mRNA and the mitochondrial encoded 12S ribosomal RNA to confer RNA stability and high total protein expression.	3880-4174
poly(A)	A 110-nucleotide poly(A)-tail consisting of a stretch of 30 adenosine residues, followed by a 10-nucleotide linker sequence and another 70 adenosine residues.	4175-4284

Sequence / Séquence / Secuencia

GAGAAΨAAAC ΨAGΨAΨΨCΨΨ CΨGGΨCCCCA CAGACΨCAGA GAGAACCCGC 50
CACCAΨGΨΨC GΨGΨΨCCΨGG ΨGCΨGCΨGCC ΨCΨGGΨGΨCC AGCCAGΨGΨG 100
ΨGAACCΨGAC CACCAGAACA CAGCΨGCCΨC CAGCCΨACAC CAACAGCΨΨΨ 150
ACCAGAGGCG ΨGΨACΨACCC CGACAAGGΨG ΨΨCAGAΨCCA GCGΨGCΨGCA 200
CΨCΨACCCAG GACCΨGΨΨCC ΨGCCΨΨΨCΨΨ CAGCAACGΨG ACCΨGGΨΨCC 250
ACGCCAΨCCA CGΨGΨCCGGC ACCAAΨGGCA CCAAGAGAΨΨ CGACAACCCC 300
GΨGCΨGCCCΨ ΨCAACGACGG GGΨGΨACΨΨΨ GCCAGCACCG AGAAGΨCCAA 350
CAΨCAΨCAGA GGCΨGGAΨCΨ ΨCGGCACCAC ACΨGGACAGC AAGACCCAGA 400
GCCΨGCΨGAΨ CGΨGAACAAC GCCACCAACG ΨGGΨCAΨCAA AGΨGΨGCGAG 450
ΨΨCCAGΨΨCΨ GCAACGACCC CΨΨCCΨGGGC GΨCΨACΨACC ACAAGAACAA 500
CAAGAGCΨGG AΨGGAAAGCG AGΨΨCCGGGΨ GΨACAGCAGC GCCAACAACΨ 550
GCACCΨΨCGA GΨACGΨGΨCC CAGCCΨΨΨCC ΨGAΨGGACCΨ GGAAGGCAAG 600
CAGGGCAACΨ ΨCAAGAACCΨ GCGCGAGΨΨC GΨGΨΨΨAAGA ACAΨCGACGG 650
CΨACΨΨCAAG AΨCΨACAGCA AGCACACCCC ΨAΨCAACCΨC GΨGCGGGAΨC 700
ΨGCCΨCAGGG CΨΨCΨCΨGCΨ CΨGGAACCCC ΨGGΨGGAΨCΨ GCCCAΨCGGC 750
AΨCAACAΨCA CCCGGΨΨΨCA GACACΨGCΨG GCCCΨGCACA GAAGCΨACCΨ 800
GACACCΨGGC GAΨAGCAGCA GCGGAΨGGAC AGCΨGGΨGCC GCCGCΨΨACΨ 850
AΨGΨGGGCΨA CCΨGCAGCCΨ AGAACCΨΨCC ΨGCΨGAAGΨA CAACGAGAAC 900
GGCACCAΨCA CCGACGCCGΨ GGAΨΨGΨGCΨ CΨGGAΨCCΨC ΨGAGCGAGAC 950
AAAGΨGCACC CΨGAAGΨCCΨ ΨCACCGΨGGA AAAGGGCAΨC ΨACCAGACCA 1000
GCAACΨΨCCG GGΨGCAGCCC ACCGAAΨCCA ΨCGΨGCGGΨΨ CCCCAAΨAΨC 1050
ACCAAΨCΨGΨ GCCCCΨΨCGG CGAGGΨGΨΨC AAΨGCCACCA GAΨΨCGCCΨC 1100
ΨGΨGΨACGCC ΨGGAACCGGA AGCGGAΨCAG CAAΨΨGCGΨG GCCGACΨACΨ 1150
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CΨΨCGΨGAΨC CGGGGAGAΨG AAGΨGCGGCA GAΨΨGCCCCΨ GGACAGACAG 1300
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GΨGAΨΨGCCΨ GGAACAGCAA CAACCΨGGAC ΨCCAAAGΨCG GCGGCAACΨA 1400

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CAAΨΨACCΨG ΨACCGGCΨGΨ ΨCCGGAAGΨC CAAΨCΨGAAG CCCΨΨCGAGC 1450
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GΨGGAAGGCΨ ΨCAACΨGCΨA CΨΨCCCACΨG CAGΨCCΨACG GCΨΨΨCAGCC 1550
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GCCGGGAΨAΨ CGCCGAΨACC ACAGACGCCG ΨΨAGAGAΨCC CCAGACACΨG 1800
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CCAACΨΨCAC CAΨCAGCGΨG ACCACAGAGA ΨCCΨGCCΨGΨ GΨCCAΨGACC 2250
AAGACCAGCG ΨGGACΨGCAC CAΨGΨACAΨC ΨGCGGCGAΨΨ CCACCGAGΨG 2300
CΨCCAACCΨG CΨGCΨGCAGΨ ACGGCAGCΨΨ CΨGCACCCAG CΨGAAΨAGAG 2350
CCCΨGACAGG GAΨCGCCGΨG GAACAGGACA AGAACACCCA AGAGGΨGΨΨC 2400
GCCCAAGΨGA AGCAGAΨCΨA CAAGACCCCΨ CCΨAΨCAAGG ACΨΨCGGCGG 2450
CΨΨCAAΨΨΨC AGCCAGAΨΨC ΨGCCCGAΨCC ΨAGCAAGCCC AGCAAGCGGA 2500
GCΨΨCAΨCGA GGACCΨGCΨG ΨΨCAACAAAG ΨGACACΨGGC CGACGCCGGC 2550
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CCGAΨGAGAΨ GAΨCGCCCAG ΨACACAΨCΨG CCCΨGCΨGGC CGGCACAAΨC 2700
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AGAGGAACΨG GACAAGΨACΨ ΨΨAAGAACCA CACAAGCCCC GACGΨGGACC 3550
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ΨCΨGGCΨGGG CΨΨΨAΨCGCC GGACΨGAΨΨG CCAΨCGΨGAΨ GGΨCACAAΨC 3750
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CGCAAΨGCΨA GCΨGCCCCΨΨ ΨCCCGΨCCΨG GGΨACCCCGA GΨCΨCCCCCG 395
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ACCΨCGGGΨC CCAGGΨAΨGC ΨCCCACCΨCC ACCΨGCCCCA CΨCACCACCΨ 4000
CΨGCΨAGΨΨC CAGACACCΨC CCAAGCACGC AGCAAΨGCAG CΨCAAAACGC 4050
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ΨAAACGAAAG ΨΨΨAACΨAAG CΨAΨACΨAAC CCCAGGGΨΨG GΨCAAΨΨΨCG 4150
ΨGCCAGCCAC ACCCΨGGAGC ΨAGCAAAAAA AAAAAAAAAA AAAAAAAAAA 4200
AAAAGCAΨAΨ GACΨAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 4250
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAA                   4284
```

Ψ = 1-methyl-3'-pseudouridylyl

The modified vaccine RNA sequence is 4,284 nucleotides long. [125] It consists of a 5'-cap; a 5' untranslated region derived from the human alpha globin sequence[126] ; a signal peptide (bases 55-102); and two proline substitutions (K986P and V987P, designated "2P." The 2P proline substitutions in spike proteins were originally developed for a MERS vaccine) that cause the spike to adopt a stabilized conformation at pre-fusion that increases its expression and stimulates the production of neutralizing antibodies[127] ; a gene optimized for the full-length spike protein codon of SARS-CoV-2 (bases 103-3879) followed by a combined 3' untranslated region (bases 3880- 4174) of AES and mtRNR1 selected for enhanced protein expression and mRNA stability[128] and a poly (A) tail comprising 30 adenosine residues, a 10-nucleotide linker sequence and 70 other adenosine residues (bases 4175-4284).
The sequence contains no uridine residues, which are replaced by 1-methyl-3'-pseudouridine.

Insight

A Mechanist's Guide to the Coronavirus Genome

Whole genome sequence of SARS-Cov-2 Wuhan-Hu-1 isolate

[129]https://benchling.com/s/seq-28k9llmwnY475iv7ogwF/edit
https://www.ncbi.nlm.nih.gov/nuccore/MN908947.3

[125] World Health Organization. "Messenger RNA encoding the full-length SARS-CoV-2 spike glycoprotein.

[126] Asrani KH, Farelli JD, Stahley MR, Miller RL, Cheng CJ, Subramanian RR, Brown JM. Optimization of mRNA untranslated regions for improved expression of therapeutic mRNA. RNA Biol. 2018;15(6):756-762. doi: 10.1080/15476286.2018.1450054. Epub 2018 Mar 26.
https://www.tandfonline.com/doi/full/10.1080/15476286.2018.1450054

[127] Walsh EE, Frenck RW Jr, Falsey AR, et al.
Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates.
N Engl J Med. 2020;383(25):2439-2450. doi:10.1056/NEJMoa2027906
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7583697/

Pallesen J, Wang N, Corbett KS, et al.
Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen.
Proc Natl Acad Sci U S A. 2017;114(35):E7348-E7357. doi:10.1073/pnas.1707304114
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[128] Orlandini von Niessen AG, Poleganov MA, Rechner C, et al.
Improving mRNA-Based Therapeutic Gene Delivery by Expression-Augmenting 3' UTRs Identified by Cellular Library Screening.
Mol Ther. 2019;27(4):824-836. doi:10.1016/j.ymthe.2018.12.011
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453560/

mRNA modification and delivery strategies toward the establishment of a platform for safe and effective gene therapy
Oliwia Andries
https://biblio.ugent.be/publication/5914818/file/5914827.pdf

[129] Wu F, et al
A new coronavirus associated with human respiratory disease in China.
Nature. 2020 Mar;579(7798):265-269. doi: 10.1038/s41586-020-2008-3. Epub 2020 Feb 3. Erratum in: Nature. 2020 Apr;580(7803):E7.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7094943/

Lu R, Zhao X, Li J, et al.
Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding.
Lancet. 2020;395(10224):565-574. doi:10.1016/S0140-6736(20)30251-8
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7159086/

Chen L, Liu W, Zhang Q, et al.
RNA based mNGS approach identifies a novel human coronavirus from two individual pneumonia cases in 2019 Wuhan outbreak.
Emerg Microbes Infect. 2020;9(1):313-319. Published 2020 Feb 5. doi:10.1080/22221751.2020.1725399
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7033720/

Chan JF, Kok KH, Zhu Z, et al.
Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan
[published correction appears in Emerg Microbes Infect. 2020 Dec;9(1):540]. Emerg Microbes Infect. 2020;9(1):221-236. Published 2020 Jan 28.
doi:10.1080/22221751.2020.1719902.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7067204/

Culture, isolation and identification of SARS-Cov-2 [130]

SARS-CoV-2 Viral Culturing at CDC
Isolation, Sequence, Infectivity, and Replication Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2 Severe Acute Respiratory Syndrome Coronavirus 2 from Patient with Coronavirus Disease, United States

Insight **Protein Synthesis**

The central dogma of molecular biology
Protein synthesis
Prof. Giorgio Sartor Protein
Synthesis Translation: DNA to mRNA
to Protein
Video Life Science - Protein synthesis (Translation)

The genetic code has some important features:

- is **redundant**: many codons are synonymous, that is, they indicate the same amino acid.
- is **unambiguous**: an amino acid can be encoded by more than one triplet, but each triplet always encodes only one amino acid.
- is **universal**: each triplet always codes for one amino acid in all organisms.

With four possible "letters" (the AGCT bases for DNA and AGCU for RNA), 64 (4^3) three-letter words (the codons) can be written, but the amino acids specified by these codons are only 20.
AUG, which encodes methionine, is also the *start codon*, the signal that initiates translation.
Three codons (UAA, UAG, UGA) function as translation termination signals, or *stop codons*; when the translation device reaches one of these codons, translation stops and the polypeptide detaches from the translation complex.
With the start and stop codons removed, 60 codons remain, many more than are strictly necessary to encode the other 19 amino acids: in fact, almost all amino acids correspond to multiple codons.

[130] Nyayanit DA, Sarkale P, Baradkar S, et al.
Transcriptome & viral growth analysis of SARS-CoV-2-infected Vero CCL-81 cells
[published online ahead of print, 2020 Jul 30]. Indian J Med Res. 2020;10.4103/ijmr.IJMR_2257_20. doi:10.4103/ijmr.IJMR_2257_20
https://pubmed.ncbi.nlm.nih.gov/32773420/

Banerjee A, Nasir JA, Budylowski P, et al.
Isolation, Sequence, Infectivity, and Replication Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2.
Emerg Infect Dis. 2020;26(9):2054-2063. doi:10.3201/eid2609.201495
https://www.researchgate.net/publication/340603961_Isolation_sequence_infectivity_and_replication_kinetics_of_SARS-CoV-2

Liu Z, Zheng H, Lin H, et al.
Identification of Common Deletions in the Spike Protein of Severe Acute Respiratory Syndrome Coronavirus 2.
J Virol. 2020;94(17):e00790-20. Published 2020 Aug 17. doi:10.1128/JVI.00790-20.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7431800/pdf/JVI.00790-20.pdf

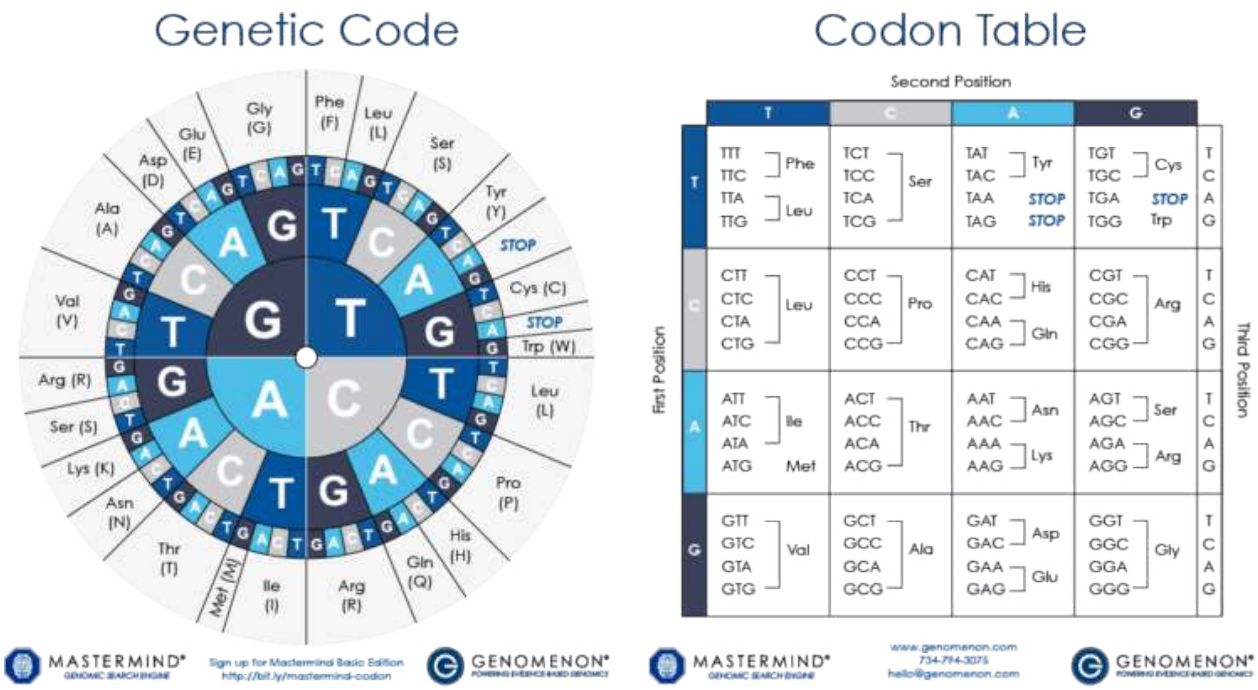
Ogando NS, Dalebout TJ, Zevenhoven-Dobbe JC, et al.
SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology
[published online ahead of print, 2020 Jun 22]. J Gen Virol. 2020;10.1099/jgv.0.001453. doi:10.1099/jgv.0.001453
https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.001453/sidebyside

Milewska A, Kula-Pacurar A, Wadas J, et al.
Replication of Severe Acute Respiratory Syndrome Coronavirus 2 in Human Respiratory Epithelium.
J Virol. 2020;94(15):e00957-20. Published 2020 Jul 16. doi:10.1128/JVI.00957-20.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7375387/

This is why the codon is said to be degenerate (i.e., it is meant to be redundant). For example, leucine is represented by six different codons. Only methionine and tryptophan are represented by a single codon each. In addition, degeneracy is not uniform, but there is preference for the use of certain codons (codon usage bias, e.g., leucine and serine have 6 codons, glycine and alanine have 4, glutamate, histidine and a tyrosine have 2). [131]

Codons are *synonymous* when they code for the same amino acid despite having a different triplet nucleotide sequence, while they are *nonsynonymous* when mutation of a nucleotide in the triplet sequence leads to the insertion of a different amino acid in the final protein.

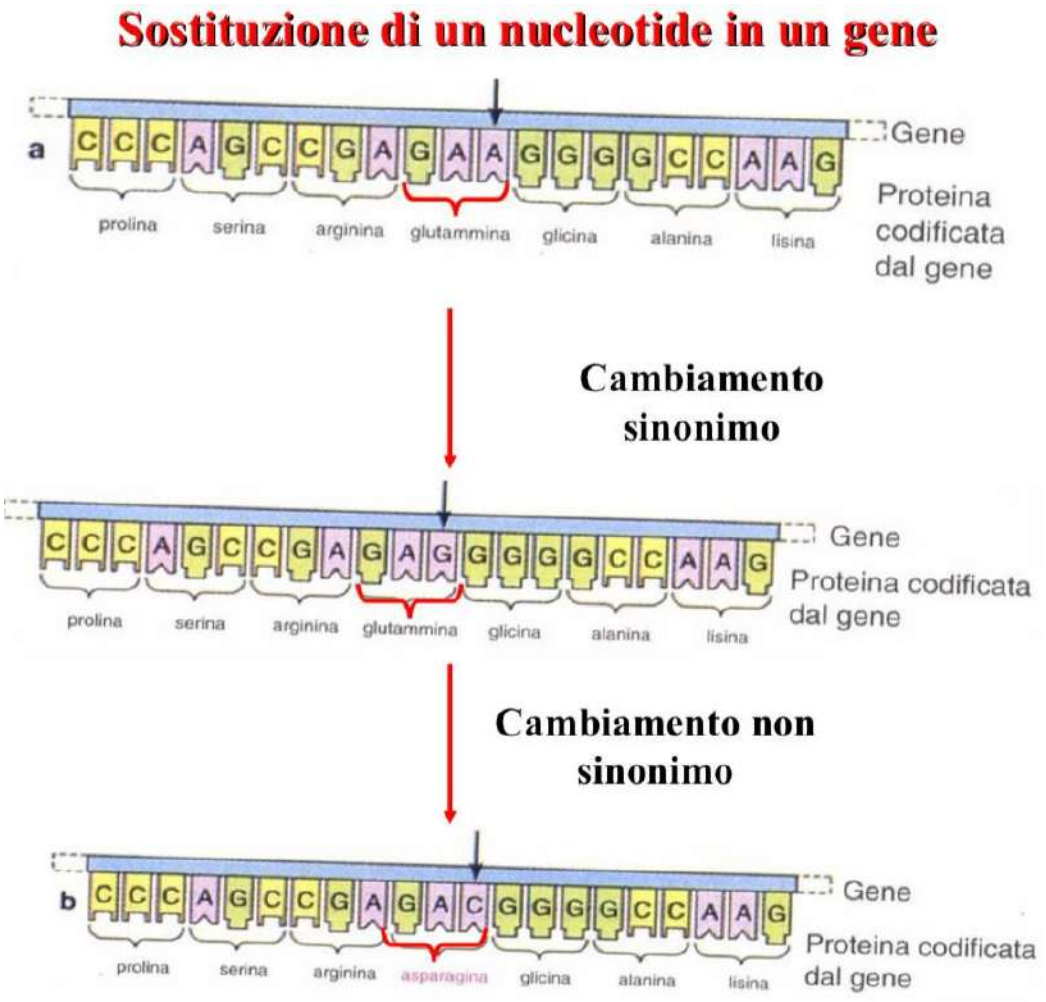
When an amino acid has multiple codons, the difference between codons generally concerns the 3ª base:

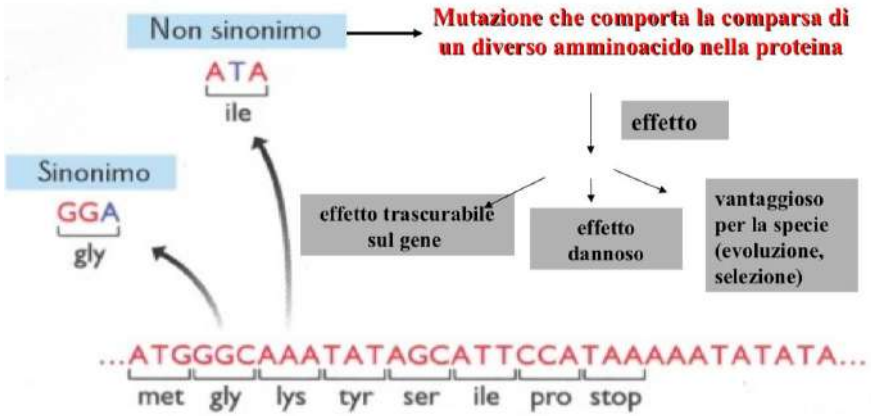


https://www.genomenon.com/codon-chart/

Translation 2

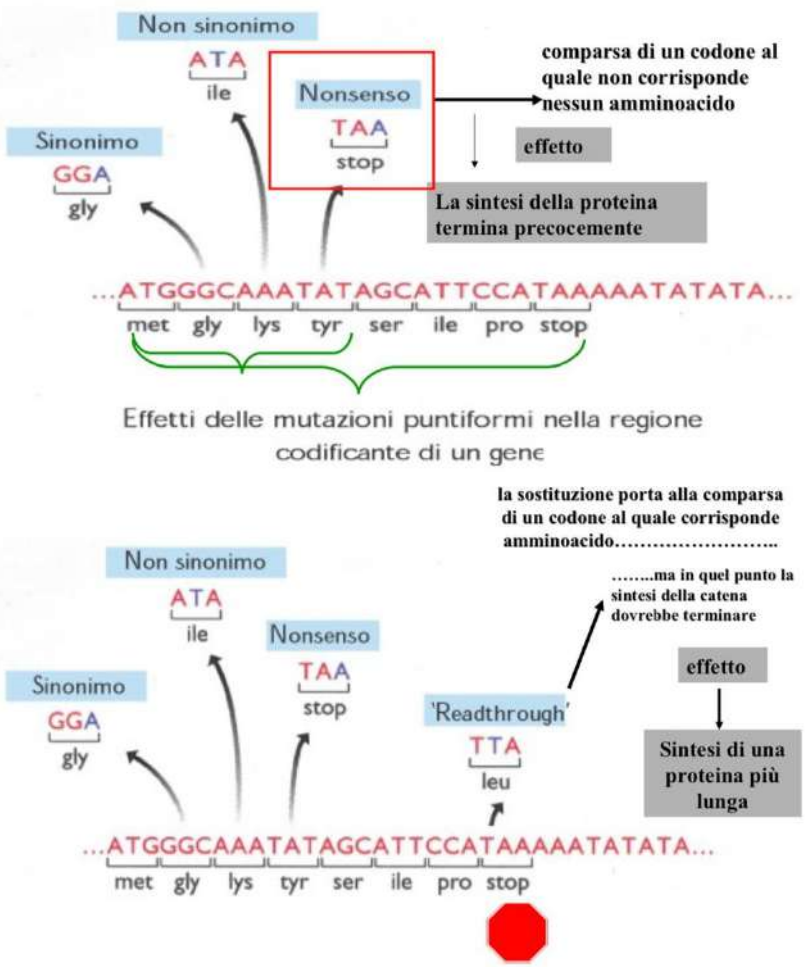
[131] Translation 2
http://unica2.unica.it/biotecnologie/index2.php?option=com_docman&task=doc_view&gid=377&Itemid=218





Effetti delle mutazioni puntiformi nella regione codificante di un gene

Translation 2



Translation 2

Gene mutations [132]

Gene mutations are nucleotide sequence variations and are divided into *base substitution* or insertion/duplication/deletion mutations also called *frameshift mutations*.

https://sites.google.com/site/mutazionigenetiche/mutazioni-geniche

[132] https://sites.google.com/site/mutazionigenetiche/mutazioni-geniche

https://www.docenti.unina.it/webdocenti-be/allegati/materiale-didattico/664022

Point mutation

A point mutation is a change in DNA sequence affecting one or a few nucleotides, but mutations of up to 50 nucleotides can also be considered "point mutations."
Many point mutations are probably without effect, in which case they are said to be *neutral*; in fact, much of the DNA in a eukaryotic genome does not code for protein products, and it is not yet known whether, and if so how, the change of a single nucleotide base in this silent part of the DNA can affect the health of an organism (it is known, however, that noncoding regions play a very important role in epigenetic regulation of DNA).
However, a single point mutation in a coding region can have a major impact on the phenotype, as is the case, for example, in sickle cell anemia.

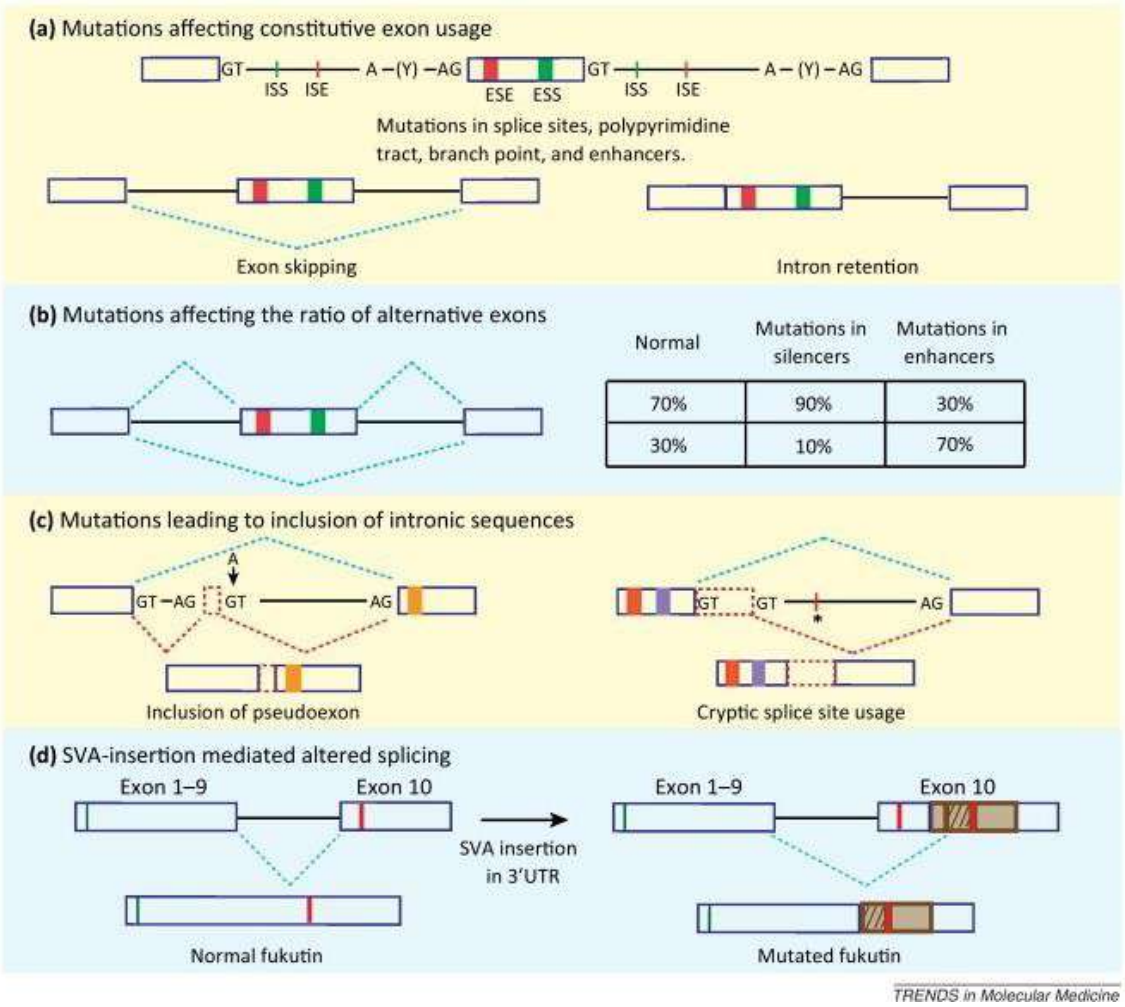
Substitution of bases

Mutations by base substitution result in the exchange of one nucleotide for another.
Transitions are called transversions if there is an exchange of a purine for another purine (A > G) or a pyrimidine for another pyrimidine (C > T); conversely, *transversions are* said to occur when the exchange is of a purine for a pyrimidine or vice versa (C/T > A/G). In general, transitions are more frequent than transversions.
Point mutations can be of six types: silent, missense, deletions or insertions in frames, nonsense insertions, frame-shift mutations or splicing mutations.

- ***Silent*** or ***synonymous mutations*** occur when the substitution of a nitrogenous base in a DNA sequence does not result in a change in the amino acid sequence of the affected protein.
  If, for example, the TTT triplet mutates to TTC, there will be a transition (T > C) in the third position of the triplet, but the amino acid encoded from the corresponding mRNA triplet (UUC) will always be phenylalanine because of redundancy in our genetic code.
  Silent mutations are mostly *neutral* because the amino acid does not change and consequently neither does the functionality of the encoded protein within which the mutated triplet is located.
  Many of the mutations responsible for altered splicing occur in the short ESE (Exon Splicing Enhancer) sequences of certain exons, which are critical for proper splicing, since certain proteins involved in the regulation of this process bind there.
  When mutations occur in these sequences, inclusion of introns in mature mRNA can occur, which, if encoded, would lead to abnormal proteins.
  Silent mutations in ESS (Exonic Splicing Silencer) sequences, which are also involved in the splicing mechanism of the primary transcript, can instead lead to the exclusion of an exon from mature mRNA and consequently to the encoding of truncated proteins by ribosomes.[133]

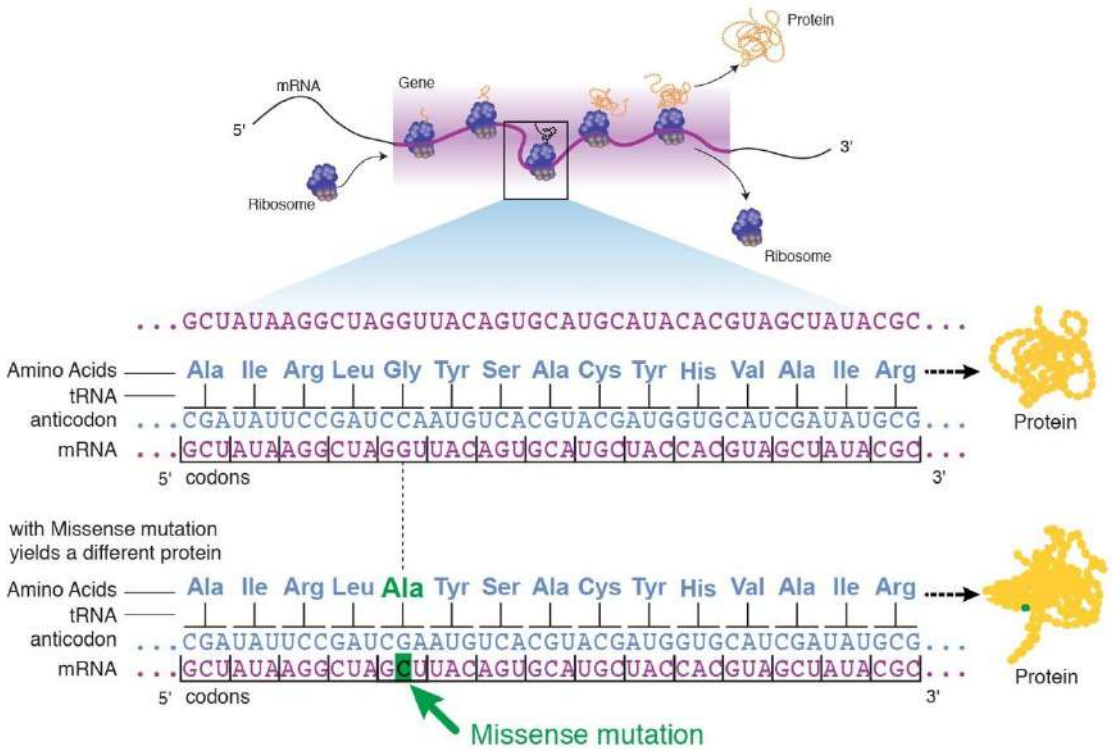
Four mechanistic categories of gene function altered by splicing mutations**. (a) The** basic cis elements of the splicing code are indicated: 5′ and 3′ splice sites (represented by GT and AG), polypyrimidine tract (Y), branch point (A), and exonic and intronic enhancers (ESE and ISE) and silencers (ESS and ISS). Mutations affecting splice sites, polypyrimidine tract, branch point or splice enhancers lead to exon skipping or intron retention. **(b)** Mutations in enhancer or silencer elements can change the ratio of isoforms containing alternative exons. **(c**) Mutations within introns can lead to the inclusion of intronic sequences (indicated by red dashed rectangles) creating a splice site/pseudo-exon (indicated by an arrow) and/or creating an enhancer element (indicated by an asterisk), allowing recognition of a cryptic splice site. The blue dashed lines indicate the normal splice pattern, while the red dashed lines indicate the splice pattern caused by mutation. **(d)** Insertion of transposable elements (SVAs, represented by a brown rectangle) into the 3′ UTR of the fukutin gene leads to the alternative use of splice sites that produce a protein with a different carboxy-terminal sequence (patterned brown rectangle). The green lines indicate the start codon of both normal and mutated fukutin, and the red lines indicate the stop codons.

[133] Singh RK, Cooper TA.
Pre-mRNA splicing in disease and therapeutics.
Trends Mol Med. 2012;18(8):472-482. doi:10.1016/j.molmed.2012.06.006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3411911/pdf/nihms388502.pdf



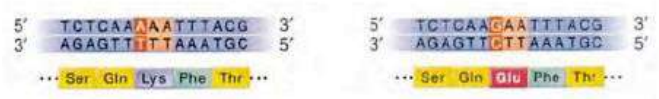
https://www.sciencedirect.com/science/article/abs/pii/S1471491412001013

- ***Missense mutations*** occur when a nitrogenous base is substituted within a DNA sequence such that the amino acid sequence is changed.
  If, for example, the TTT triplet mutates to TCT, with a base transition at the second position (T > C), the encoded amino acid will no longer be phenylalanine but serine.
  This type of mutation can be neutral and result in no specific phenotype by simply representing a single nucleotide polymorphism (SNP) or a private variant, but it can also give rise to serious diseases such as sickle cell anemia and multiple rare genetic diseases.
  Generally, a missense mutation can be considered neutral if the substituted amino acid is present without showing a pathological phenotype in a given number of individuals in the form of a single nucleotide polymorphism or private variant, or if the encoded amino acid has similar properties to the original one (e.g., a substitution of glutamic acid for aspartic acid).
  However, the mutation can give rise to pathological conditions when the amino acid encoded by the new triplet has very different properties from the previous one (e.g., the substitution of a valine for aspartic acid), when it has not been found in previous cases or in the parental environment, or when it occurs in a highly conserved region of a protein. In fact, often even a single mutation in a highly conserved region of a protein causes it to lose functionality.

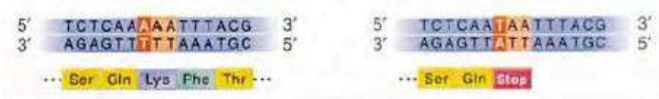


https://www.genome.gov/genetics-glossary/Missense-Mutation

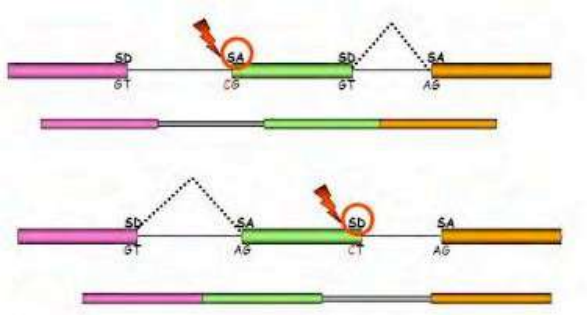
- ***Nonsense mutations*** occur when a mutation at one nucleotide of a triplet results in the transformation of a codon encoding an amino acid into a stop codon.
  For example, the TGC triplet coding for cysteine is replaced by TGA, which will be transcribed in the mRNA as UGA, one of the three stop codons.
  The consequence is that the encoded protein is either not exported or, if encoded, is truncated, as translation ends at the stop codon while ignoring its downstream triplets, leading to a truncated nonfunctional or harmful protein.
  However, if the stop codon is at least 50 nucleotides away from the closest splicing sequence in the mRNA, the cell activates a protective mechanism known as NMD (Nonsense Mediated Decay) that degrades the mutated mRNA.
  Alternatively, another mechanism known as NAS (Nonsense-associated Altered Splicing) may be activated, which excludes the exon containing the mutated triplet into a stop codon, allowing the other exons to bind into a shorter protein.



- **Mutazioni missenso**: la sostituzione nucleotidica cambia il codone per un amminoacido (aa) in un codone per un altro aa (sostituzione aacidica). Possono essere **sinonime** o **non-sinonime**



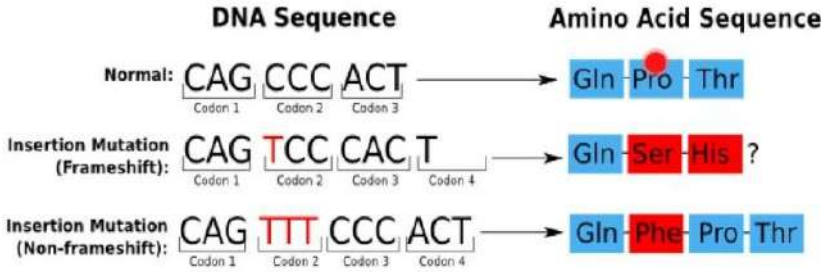
- **Mutazioni non senso**: la sostituzione nucleotidica cambia il codone per un aa in un codone di stop (proteina tronca)



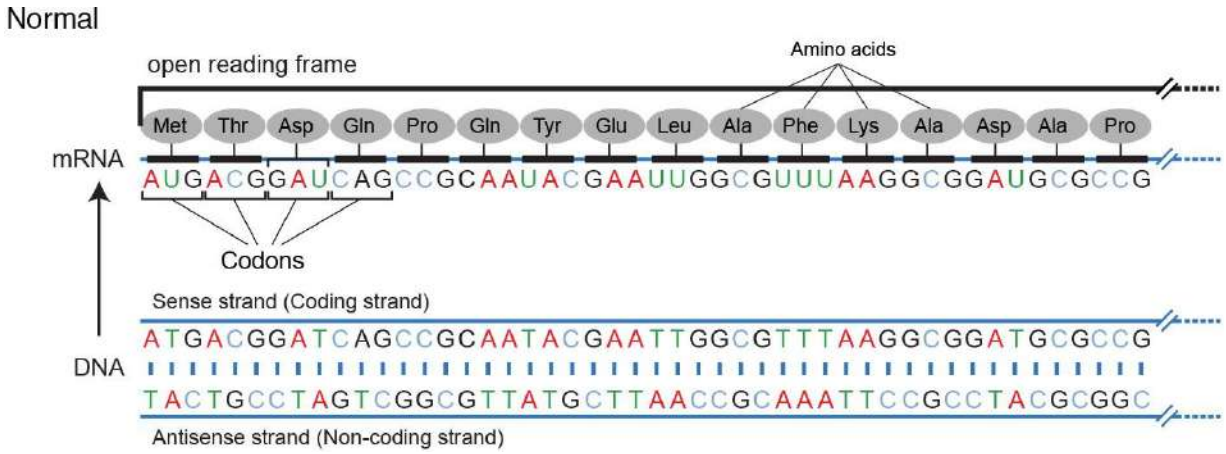
- Mutazioni che alterano trascrizione e processamento dell'RNA: alterano i siti di splicing, la stabilita' dell'mRNA (nelle regioni trascritte e non tradotte al 5' o 3') oppure la trascrizione (nel promotore)

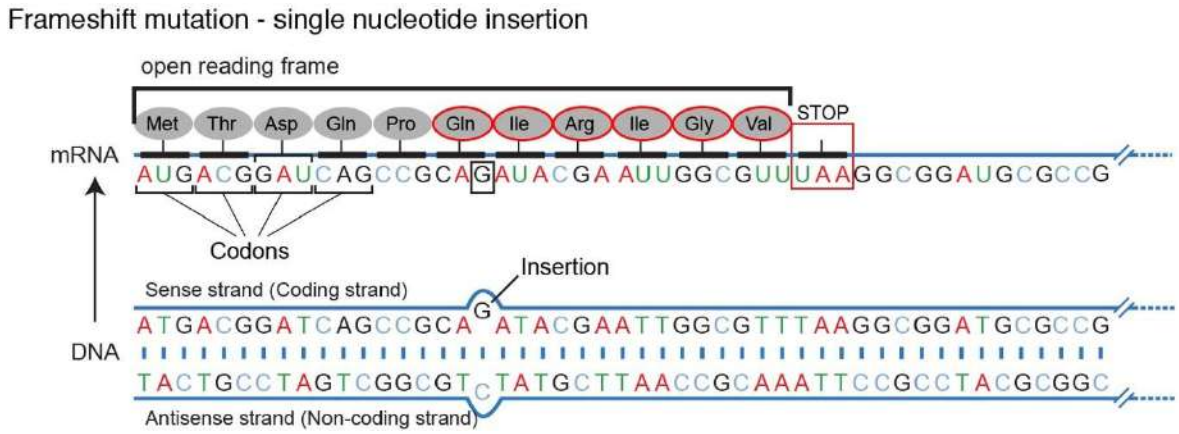
https://sites.google.com/site/mutazionigenetiche/mutazioni-geniche

- ***In-frame deletions*** and ***in-frame insertions*** result in the deletion of a triplet or number of nucleotides divisible by 3 or the insertion of a triplet or number of nucleotides divisible by 3, respectively. They are "in frame" because they do not displace the reading frame at the ribosomal level, which instead
  would result in the almost complete change of the amino acid sequence of a protein.
  These types of mutations result in the deletion or addition of amino acids in the protein encoded from the mature mRNA containing them. The consequences of these mutations are very varied.



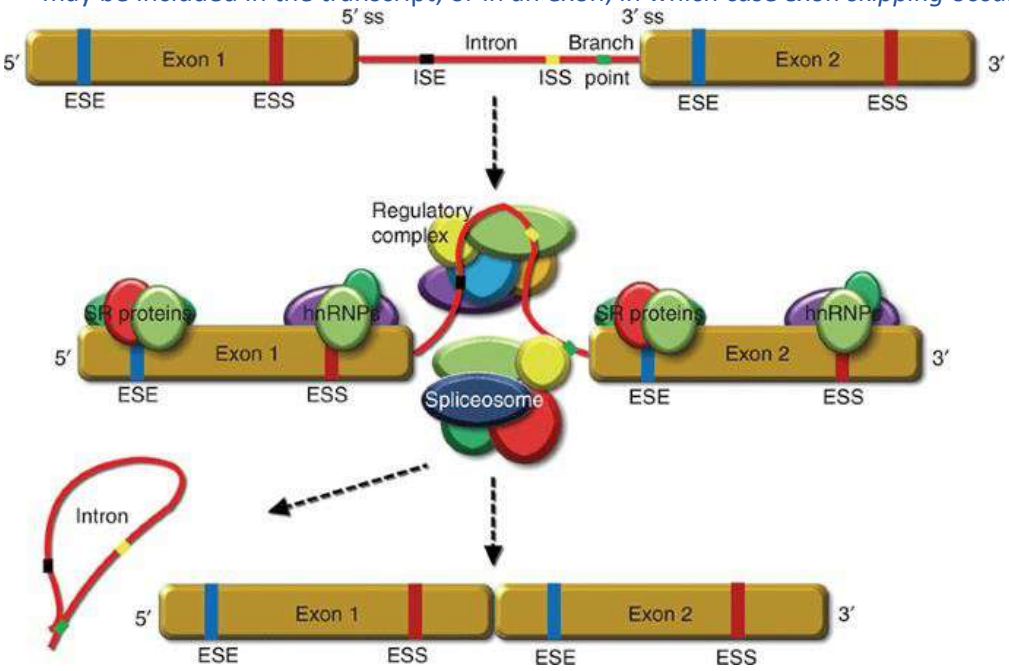
- ***Frame-shift mutations*** are due to deletions or insertions of a number of nucleotides that are not divisible by 3; this results in a shift of the reading frame downstream of the mutation and thus the encoding of an amino acid sequence that does not match that of the original transcript.
  The consequence is the production of abnormal proteins that have only sequence portions corresponding to the original or failure to export or translate the mutated mRNA.





https://www.genome.gov/genetics-glossary/Frameshift-Mutation

- ***Splicing mutations*** are a set of four types of mutations involving sequences important for pre-mRNA splicing.
  A first type involves the splicing donor site (GT) or the acceptor site (usually AG).
  Mutations in these two initial and final markers of an intronic sequence can lead to inclusion of the intron in the mature transcript or to incorrect splicing.
  A second type involves short consensus sequences upstream and downstream of the donor site and acceptor site, or a consensus sequence of the bifurcation site (*branch-site*).
  A third type involves mutations in an ESE or ESS sequence and can also be ascribed to silent mutations. Finally, a last type involves mutations that create new consensus sequences within an intron, in which case it or parts of it may be included in the transcript, or in an exon, in which case *exon skipping* occurs.[134]



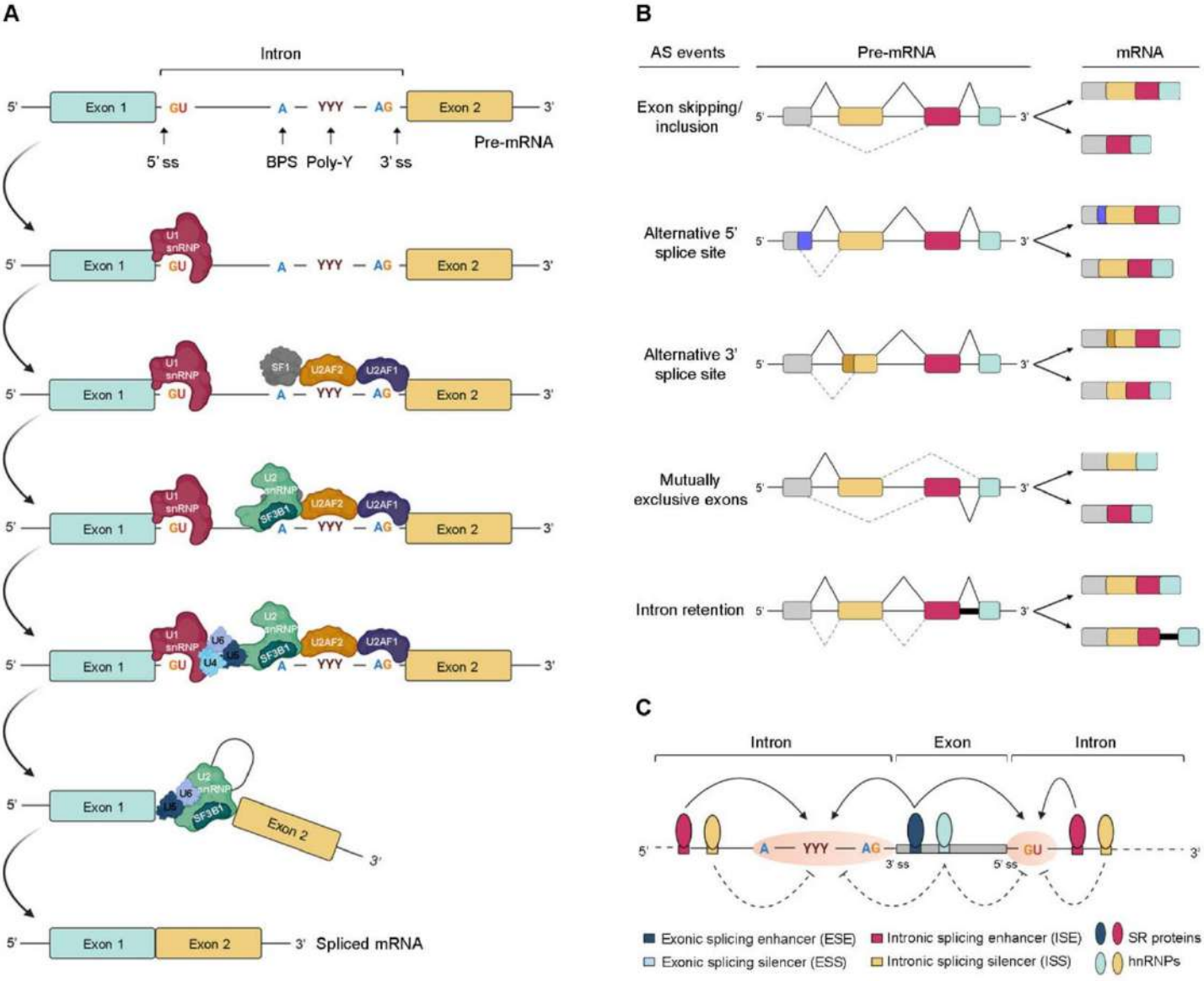
Schematic illustration of pre-mRNA splicing. Splice site 5 ′ and splice site 3 ′ are recognized by the spliceosome, the intron is excised, and the exons are soldered. The whole process is regulated by trans-active elements such as SR proteins, heterogeneous nuclear ribonucleoproteins and the regulatory complex. ESE, exonic splicing enhancer (enhancer); ESS, exonic splicing silencer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; ss, splice site

[134] Bessa C, Matos P, Jordan P, Gonçalves V.
Alternative Splicing: Expanding the Landscape of Cancer Biomarkers and Therapeutics.
Int J Mol Sci. 2020 Nov 27;21(23):9032. doi: 10.3390/ijms21239032.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7729450/

Ule J, Blencowe BJ.
Alternative Splicing Regulatory Networks: Functions, Mechanisms, and Evolution.
Mol Cell. 2019 Oct 17;76(2):329-345. doi: 10.1016/j.molcel.2019.09.017.
https://www.cell.com/action/showPdf?pii=S1097-2765%2819%2930702-6

Jian, X., Boerwinkle, E. & Liu, X.
In silico tools for splicing defect prediction: a survey from the viewpoint of end users.
Genet Med 16, 497-503 (2014). https://doi.org/10.1038/gim.2013.176
https://www.nature.com/articles/gim2013176

https://www.nature.com/articles/gim2013176



https://www.mdpi.com/1422-0067/21/23/9032/htm

Regulation of pre-mRNA splicing. (A) Stepwise assembly of the spliceosome onto the pre-mRNA and catalysis of the splicing reaction to generate mature spliced mRNA. (B) Schematic representation of the most common alternative splicing AS events. The gray, yellow, red and blue boxes represent different exons. The dotted black and gray lines indicate distinct splicing events. (C) Complex interaction between cis- and trans-acting factors in AS regulation. RNA- binding motif (RBM) proteins, serine/arginine-rich (SR) proteins, and heterogeneous (hn) ribonucleoproteins (hnRNPs) bind to exonic or intronic regulatory elements to promote or prevent recognition of 3' or 5' (ss) splice sites from small (sn) RNPs (snRNPs) and splicing factors. The solid and dashed black arrows represent stimulation and inhibition of binding, respectively; (ss - splicing sites; BPS - branch point site; poly-Y tract - polypyrimidine; pre-mRNA - precursor messenger RNA; snRNPs - small nuclear ribonucleoprotein particle; SF1 - splicing factor 1; U2AF - snRNP auxiliary factor U2).

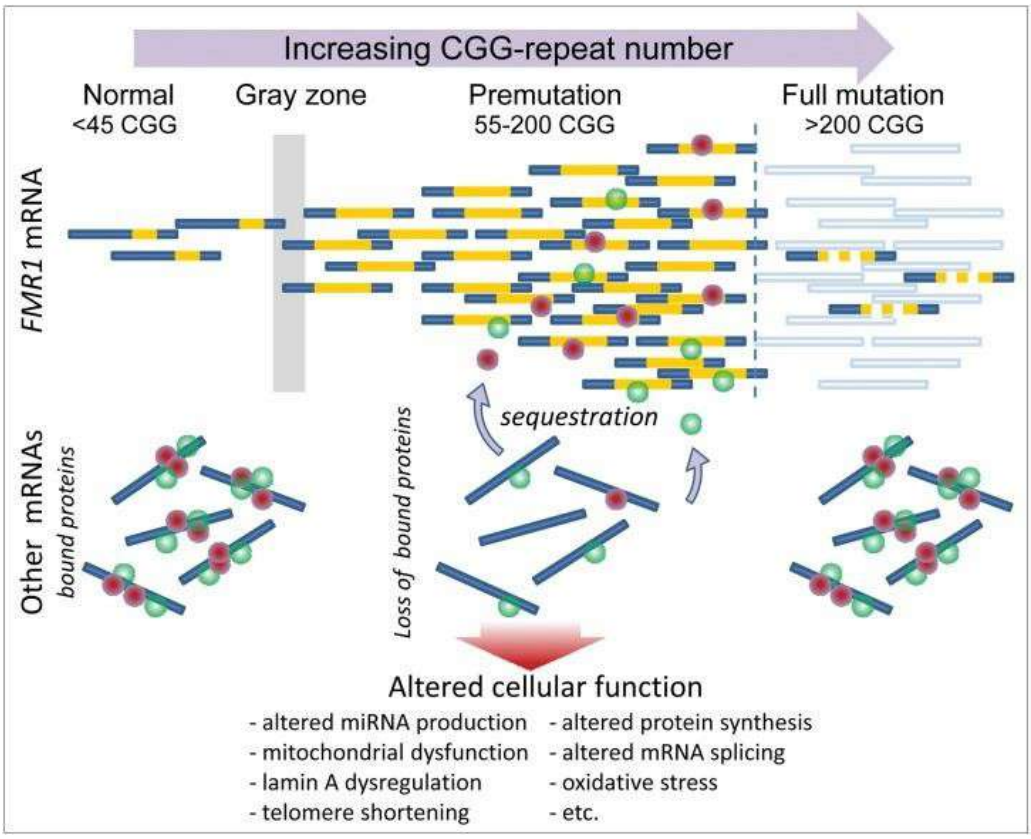
Mutations in transcription regulatory regions

Point mutations can also occur within the regulatory region of a gene. This can result in widely varying consequences ranging from no phenotypic effect to changes in gene expression that give rise to severe disease.

Dynamic Mutations

Dynamic mutations are due to the repetition of short nucleotide triplets within a coding (in this case, the most frequent triplet is CAG encoding glutamine) or noncoding region of a gene. The mutation that originates in the course of DNA replication causes a change in the number of these repeated sequences; the new strand of DNA may have excess or deficient sequences. The phenomenon that causes the mutation is called *replication slippage* and is due to the mismatch of the two strands

complementary. Genetic diseases associated with this type of mutation are Huntington's Korea and Fragile X syndrome.[135]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3379894/ Overview of FMR1 gene expression. (Fragile X syndrome)
FMR1 mRNA levels increase with increasing length of CGG repeats (gold segments) throughout the pre-mutation interval and undergo a transition to significantly reduced levels throughout the mutation interval due to hypermethylation of the FMR1 promoter region. In some cases, methylation mosaicism results in continuous production of low to moderate mRNA levels throughout the mutation interval. RNA toxicity in the pre-mutation interval is thought to result from sequestration, from direct binding to the expanded CGG repeat element within the FMR1 mRNA, of one or more RNA-binding proteins that would normally be associated with other mRNAs. Sequestration in turn leads to loss of the normal functions of those proteins, which may include modulation of splicing and regulation of miRNA production, among other functions. Dysregulation of RNA processing is thought to lead to multiple forms of downstream cellular dysregulation

Video Gene mutations step by step

[135] Hagerman PJ.
Current Gaps in Understanding the Molecular Basis of FXTAS.
Tremor Other Hyperkinet Mov (N Y). 2012;2:tre-02-63-375-2. doi: 10.7916/D80C4TH0. Epub 2012 May 18.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3379894/

Potential dysregulation of protein synthesis with Pfizer vaccine

Dr. Kira Smith in her article "*BNT162b2 Vaccine: Possible Codons Misreading, Errors in Protein Synthesis and Alternative Splicing's Anomalies*" discusses the possible consequences of engineering the spike mRNA sequence on translation into protein of which an elaboration of the text is given: [136]

How the vaccine works

BioNTec / Pfizer's CoV-2 (Covid-19 virus) SARS vaccine called BNT162b2, but also Tozinameran, or Comirnaty, contains about 30 µg of RNA, which is injected into a lipid sphere inside the human body, specifically inside the cytoplasm of cells, but outside the nucleus (where the DNA is located); this RNA has the genetic information modified (so it is a modRNA), that is, an mRNA (messenger RNA) containing instructions to put in place a protein factory, clones of the S Spike protein, that is, the protein (and only the protein, not the whole virus) used by the Covid-19 virus to enter the host and infect it.
Once serially produced by ribosomes, they are transported outside the cell, past the lipid coating; thus the immune system identifies these proteins as cellular invaders and attacks them, through the production of antibodies.
This is why it is inconceivable that the vaccine induces Covid-19 or changes human DNA. [137]

Protein synthesis

Translation is generally divided into three stages: initiation, elongation and termination. The ribosome binds to the mRNA at the start codon; the polypeptide chain elongates in the direction of ribosomal movement by subsequent addition of amino acids; when a Stop codon is found, the polypeptide is released and the ribosome dissociates.

Errors in sequence assembly and translation

The conversion of the mRNA sequence into a polypeptide depends on transfer RNA (tRNA) to transport amino acids to the ribosome. In ribosomes, tRNA pairs with mRNA by complementary base pairing between mRNA codon nucleotides and tRNA anticodon nucleotides. Once the correct tRNA is bound to a codon, it transfers its amino acid to the end of a growing polypeptide chain.
Deciphering mRNA codons by transfer RNA (tRNA) in the ribosome involves base pairing
according to Watson-Crick.

It is estimated that the overall error rates of genomic replication (about 10^{-8}) are about 10,000 times lower than those of protein synthesis (about 10^{-4}), and thus in most cases mRNA translation is the key process contributing to the inaccuracy of the cellular proteome.
The discrepancy between error rates in DNA replication and mRNA translation may be partly related to the fact that DNA replication occurs at the level of single nucleotides (involving $4^1 = 4$ possible permutations), while the translation mechanism interprets mRNA codons into triplets (involving $4^3 = 64$ possible permutations).
The efficiency of the mRNA decoding machinery is also essentially governed by codon utilization bias, which is distinguished by over- or under-represented synonymous codons. Consequently, optimizing tRNA oscillation and codon utilization bias in mRNA can substantially improve translation efficiency and accuracy.

During transcription and post-translational processing of mRNA, errors can be introduced indirectly
in the protein sequence. However, the translation mechanism may directly contribute to the mistranslation

[136] Smith K
BNT162b2 Vaccine: Possible Codons Misreading, Errors in Protein Synthesis and Alternative Splicing's Anomalies.
J Antivir Antiretrovir. (2021) 13:210.
https://www.longdom.org/open-access/bnt162b2-vaccine-possible-codons-misreading-errors-in-protein-synthesis-and-alternative-splicings-anomalies.pdf
https://www.sanambiens.it/vaccino-covid-19-the-worst-case-scenario/

https://berthub.eu/articles/posts/italian-reverse-engineering-source-code-of-the-biontech-pfizer-vaccine/
https://berthub.eu/articles/posts/reverse-engineering-source-code-of-the-biontech-pfizer-vaccine/

[137] https://www.deplatformdisease.com/blog/no-really-mrna-vaccines-are-not-going-to-affect-your-dna
https://www.deplatformdisease.com/blog/mrna-vaccines-and-covid-19

by incorrect tRNA decoding (leading to incorrect incorporation or stop codon reading), tRNA mis-acylation (leading to incorrect amino acid coupling of the tRNA), codon reassignment or frame shifts caused by ribosomal translocation.[138]

METHOD OF INVESTIGATION

Genetic sequence analysis

The vaccine consists of a 4284-nucleotide pre-mRNA containing the entire Spike sequence of SARs-Cov-2, divided into 6 sections:

Cap is the beginning of the sequence, which opens with the two **GA** nucleotides, falsely indicating that the mRNA came from the human cell for it to be accepted;

The **5' indicates the** direction to be followed for translation, while the **UTR** indicates the area where the ribosome must reside to produce the protein (in this case an optimized sequence from the alpha globulin gene that increases the amount of protein produced).[139]

GAGAAΨAAACΨAGΨAΨCΨCΨGGΨCCACAGACΨCAGAGAACCCGCCACC

5' UTR (position 1-54)

In this segment, the U of uracil was replaced with a 1-methyl-3'-pseudouridine molecule, denoted Ψ, to bypass the immune system and prevent degradation of the newly entered mRNA; however, this is a factor that can lead to errors in protein production.

Multiple Ψ-synthases are involved in modifying specific protein positions, and in many of them the defects are linked to human diseases.[140]

Then there is the **sig** section, called *the extended initiation sequence* of the glycoprotein S signaling peptide, whose information is needed to guide the newly formed protein out of the cell through the endoplasmic reticulum.

Virus: AUG UUU GUU UUU CUU GUU UUA UUG CCA CUA GUC UCU AGU CAG UGU GUU
Vaccine:AUG UUC GUG UUC CUG GUG CUG CCU CUG GUG UCC AGC CAG UGU GUG

Ψ is replaced with U to facilitate comparison of sequences - sig sequence (position 55-102)

Here, too, nucleotide triplet modifications are put in place to make the RNA accepted by the immune system, changing some letters, which make up the information, with others (usually in 3ª position, according to the mechanism of "oscillation"), apparently "harmless synonyms" (mainly by increasing the number of letters C and G, which encode the rate of protein synthesis).

The changes in the vaccine (UUU -> UUC) are all synonymous, but RNA with more G and C is converted more efficiently to protein, probably preventing hairpin formation in the RNA [141]

[138] Ou X, Cao J, Cheng A, Peppelenbosch MP, Pan Q.
Errors in translational decoding: tRNA wobbling or misincorporation?
PLoS Genet. 2019 Mar 28;15(3):e1008017. doi: 10.1371/journal.pgen.1008017.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6438450/

[139] Asrani KH, Farelli JD, Stahley MR, Miller RL, Cheng CJ, Subramanian RR, Brown JM. Optimization of mRNA untranslated regions for improved expression of therapeutic mRNA. RNA Biol. 2018;15(6):756-762. doi: 10.1080/15476286.2018.1450054. Epub 2018 Mar 26.
https://www.tandfonline.com/doi/full/10.1080/15476286.2018.1450054

[140] Khonsari B, Klassen R.
Impact of Pus1 Pseudouridine Synthase on Specific Decoding Events in Saccharomyces cerevisiae.
Biomolecules. 2020 May 7;10(5):729. doi: 10.3390/biom10050729.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7277083/

[141] Pardi, N., Hogan, M., Porter, F. et al.
mRNA vaccines - a new era in vaccinology.
Nat Rev Drug Discov 17, 261-279 (2018). https://doi.org/10.1038/nrd.2017.243
https://www.nature.com/articles/nrd.2017.243

https://moodle2.units.it/pluginfile.php/104451/mod_forum/attachment/5811/4-Struttura%20RNA%20e%20topologia%20del%20DNA%20BS.pdf

However, while they specify identical amino acids, the two synonyms are not exactly the same, at least when it comes to the act of translation.
Mechanistic studies show that there are subtle but significant differences in the way each interacts with its corresponding transfer RNA (tRNA), differences that affect both the speed and accuracy of translation. [142]
While it is true that 3 letters form a codon and more than one codon codes for the same amino acid, it is also true that by disproportionately increasing the rate of protein production, there could be risks of serious translation errors.

The characters that make up the sequence related to the construction of the true Spike **S protein_mut were** also altered with more Cs and Gs that could be added, respecting the synonyms in the standard genetic code table, with replacement of the amino acids Lysine (AAA) and Valine (GUU) with Proline (CUU)[143] , an amino acid that tends to make the protein structure particularly rigid, to prevent the constructed protein from collapsing in on itself, nullifying the vaccine's ability to produce anti-spike antibodies[144] .
At the end of this sequence there are 2 stop codons. It is not completely proven that the same elements will form with this substitution, and there will be no misreading errors.

```
          L   D   K   V   E   A   E   V   Q   I   D   R   L   I   T   G
Viruses:  CUU GAC AAA GUU GAG GCU GAA GUG CAA AUU GAU AGG UUG AUC ACA GGC
Vaccines: CUG GAC CCU GAG GCC GUG CAG AUC GAC AGA CUG AUC ACA GGC L   D   P   P E
              A   E   V   Q   I   D   R   L   I   T   G
```

Sequence containing 2P substitution (The Ψ is replaced with U to facilitate sequence comparison) sequence S protein_mut (position 103-3879 detail 3004-3051)

```
          V   L   K   G   V   K   L   H   Y   T   s
Virus:    GUG CUC AAA GGA GUC     UUA CAU UAC ACA UAA
Vaccines: GUG CUG AAG GGC A A A   COUS CAC UAC ACA UGA UGA
          V   L   K   G   V   K   L   H   Y   T   s   s
```

Terminal sequence of the S protein_mut (The Ψ is replaced with U to facilitate sequence comparison)
The original virus uses the UAA stop codon, the vaccine uses two UGA stop codons (position 3844-3879)

3'-UTR (Untranslated Region 3 First): should indicate the direction of sequence translation and enhance protein synthesis, however, many of its functions remain unknown; therefore, it is impossible to ascertain its safety.
The only thing that is known, as stated by WHO, is the following sentence: The UTR 3' for the BioNTech / Pfizer vaccine was taken from "Amino-terminal Enhancer of Split (AES) mRNA and mitochondrial-encoded 12S ribosomal RNA."

Kudla G, Lipinski L, Caffin F, Helwak A, Zylicz M.
High guanine and cytosine content increases mRNA levels in mammalian cells.
PLoS Biol. 2006 Jun;4(6):e180. doi: 10.1371/journal.pbio.0040180. Epub 2006 May 23.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1463026/

[142] Robinson R.
Which codon synonym is best? It may depend on what's on the menu.
PLoS Biol. 2014 Dec 9;12(12):e1002014. doi: 10.1371/journal.pbio.1002014.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4260823/

[143] Pallesen J, Wang N, Corbett KS, et al.
Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen.
Proc Natl Acad Sci U S A. 2017;114(35):E7348-E7357. doi:10.1073/pnas.1707304114
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5584442/

[144] https://cen.acs.org/pharmaceuticals/vaccines/tiny-tweak-behind-COVID-19/98/i38

Poly (A): We then reach the end of the sequence and encounter 30 A, then a 10-nucleotide GCAUAUGACU linkage, followed by another 70 A.

Each mRNA can be reused by the organism multiple times, but when this happens it also loses some of the A at the end. Once the A is depleted, the mRNA is no longer functional and is discarded. [145] In this way, the "poly-A" tail is a protection from degradation.

UAGCAAAAAA AAAAAAAAAAAAAAA AAAAAAAAAA A A A A A A A A A A A A A A A A A A A A AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAA

(The Ψ is replaced with U to facilitate sequence comparison.) In yellow is the 10-nucleotide linker

The 10-nucleotide linker within the poly(A) tail appears to be able to facilitate the joining of the synthetic DNA fragments that become the mold for mRNA transcription and to reduce slippage by RNA polymerase T7, so that the transcribed mRNA is more uniform in length improving its efficiency. [146]

All these modifications to increase protein expression are patented, and nothing is known about the actual translation that takes place in the body.

Alternative splicing and other errors

Another related problem is that the same pre-mRNA can give rise to different mature mRNAs, and thus slightly different proteins (**alternative splicing** abnormalities).
An alteration in the process of protein synthesis has been found to be the cause of the development and growth of some cancers and other diseases, without altering the DNA in any way.
For example, all the splicing events identified in the three PHT (Phosphorus Transporter Genes) series genes involve the loss of the read frame of the messenger sequence and the introduction of a premature termination codon (PTC) always located more than 50-55 nucleotides upstream of the last exon-exon junction, which makes the alternative transcripts targets of the NMD (Nonsense-mediated mRNA Decay) surveillance system.
For human and rat slc15a4/ PHT1, this was demonstrated by NMD inhibition experiments in several cell lines, in which the expression of alternative variants to canonical transcripts was always stabilized after inhibition. [147]

CONCLUSIONS

Possible long-term risks to human health

The author states that in addition to not being optimized, the sequence has strong doubts about the presence of codon usage errors. It is possible to speculate that excessive alteration aimed at an extreme increase in protein expression may be a source of errors in the assembly of the mRNA gene sequence.[148]

---

[145] Jacobson A, Peltz SW.
Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells.
Annu Rev Biochem. 1996;65:693-739. doi: 10.1146/annurev.bi.65.070196.003401.
https://pubmed.ncbi.nlm.nih.gov/8811193/

Eisen TJ, Eichhorn SW, Subtelny AO, et al.
The Dynamics of Cytoplasmic mRNA Metabolism.
Mol Cell. 2020;77(4):786-799.e10. doi:10.1016/j.molcel.2019.12.005
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7265681/

[146] Trepotec Z, Geiger J, Plank C, Aneja MK, Rudolph C.
Segmented poly(A) tails significantly reduce recombination of plasmid DNA without affecting mRNA translation efficiency or half-life.
ANN. 2019;25(4):507-518. doi:10.1261/rna.069286.118
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6426288/

[147] Andries, O.
mRNA Modification and delivery strategies toward the establishment of a platform for safe and effective gene therapy.
Ghent University. Faculty of Veterinary Medicine, Merelbeke, Belgium. (2015).
https://biblio.ugent.be/publication/5914818/file/5914827.pdf

[148] Wu Q, Medina SG, Kushawah G, DeVore ML, Castellano LA, Hand JM, Wright M, Bazzini AA.

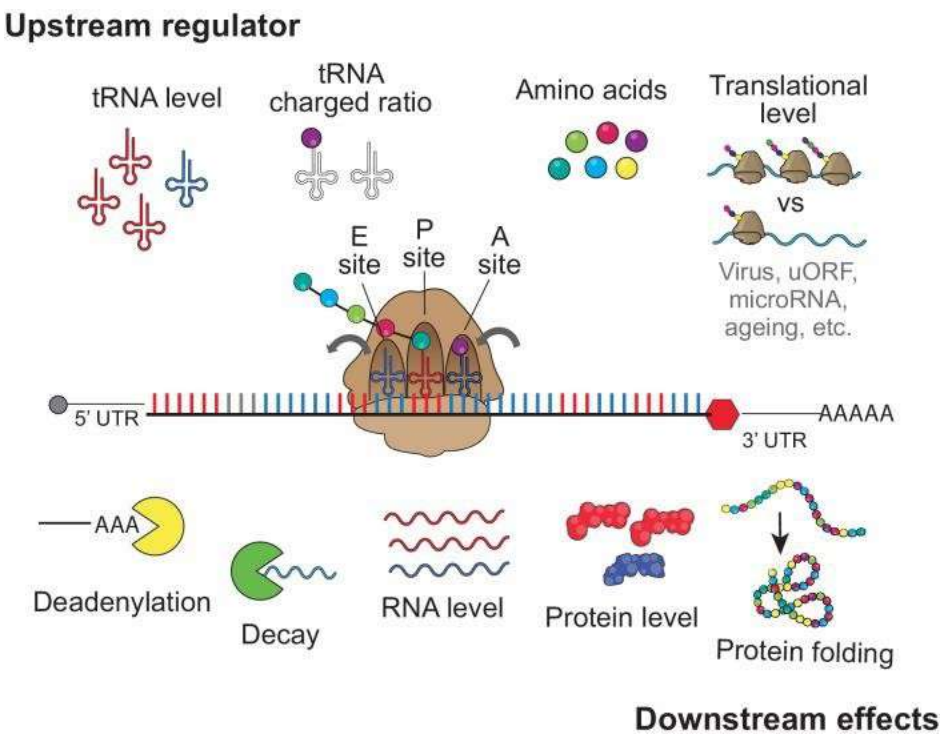


Fig. 2 the upstream regulator and downstream effect for codon optimality The level of tRNA, tRNA-loaded ratio, amino acid and translation level could contribute t o regulate the regulatory identity and/or strength of each codon to affect gene expression, influencing the rate of translation elongation. The downstream effects of the codon-mediated mechanism are RNA deadenylation, mRNA decay, mRNA level, protein level and protein folding.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6529216/

Altering the availability of tRNAs [and thus their profile, which depends on the type and state of cell differentiation], can lead to neurodegenerative diseases[149] , while upregulation of specific tRNAs drives metastasis by increasing the stability of transcripts enriched in their related codons. [150]

Mistranslation has very serious consequences on the pathophysiology of a variety of diseases, including multiple sclerosis, neurodegeneration, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes, Parkinson's disease, and cancer (genesis, growth acceleration, and metastasis).[151]

Translation affects mRNA stability in a codon-dependent manner in human cells.
Elife. 2019 Apr 23;8:e45396. doi: 10.7554/eLife.45396.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6529216/

[149] Ishimura R, Nagy G, Dotu I, Zhou H, Yang XL, Schimmel P, Senju S, Nishimura Y, Chuang JH, Ackerman SL.
RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration.
Science. 2014 Jul 25;345(6195):455-9. doi: 10.1126/science.1249749.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4281038/

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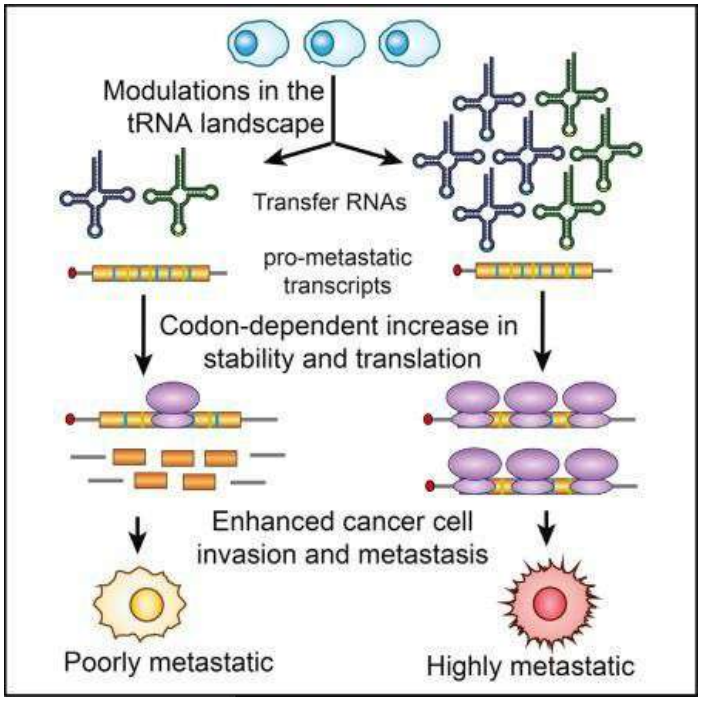

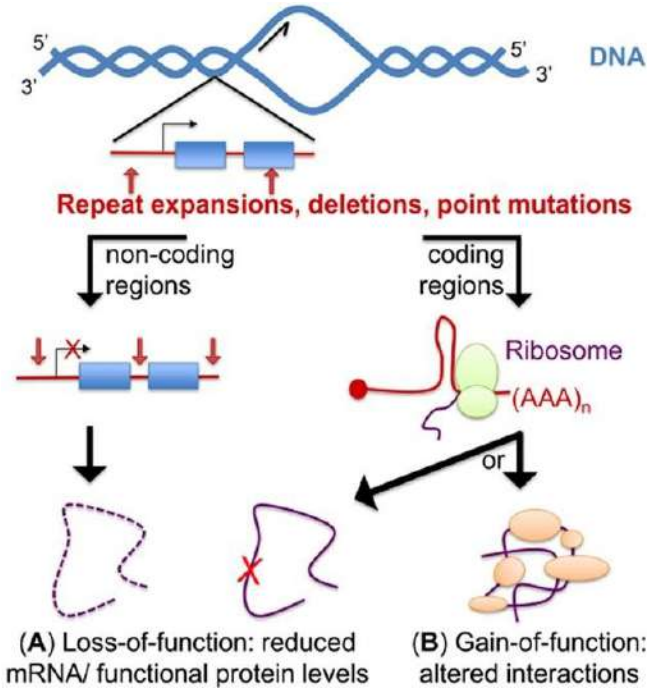

Fig1 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4915377/Fig.2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4329338/
Fig. 2 Mechanisms conferring protein loss and toxic gain-of-function effects. The diagram illustrates the pathogenic mutations (repeat expansions, deletions, point mutations) that can occur in the noncoding or coding regions of the genome (left and right sides, respectively). (A) Loss of protein function. Haploinsufficiency can occur when the level of a particular mRNA is under-regulated due to mutations in noncoding regions of genes such as promoters/introns, or if the promoter is subjected to histone/DNA modifications (transcriptional repression), but also if mutations in 5 ′ or 3 ′ untranslated regions (UTRs) reduce mRNA stability. Loss of protein function can also occur when mutations in coding regions directly alter the activity of the mutated protein (misfolding, active site alteration). (B) Gain of toxic protein function is caused by mutations in coding regions that promote abnormal interactions, increase the interaction of the mutated protein with its natural ligands, and/or promote misfolding/aggregation.

The correlation of the 100% increased rate of protein synthesis with sequence translation errors, as well as the mechanism affecting amino acid production, remain obscure here for now, as many trials have not yet been performed.
The author concludes by arguing that the total sequence code is inherently altered in an unbalanced way, too much compared to the natural viral counterpart, and too much for the human organism to be said to be able to reproduce exactly the S Spike proteins of the natural virus, thus risking serious damage to human health in the long term, in addition to ineffectiveness in immunization.

What will be produced from that sequence is far from well defined, but it is written in each individual's genes, in the ribosomal profile, how it will be translated and what will be produced, and consequently the benefits or harms that will result.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526920/
Dr. Loretta Bolgan 08.03.2021

"MODERN" VACCINE mRNA PRODUCTION. [152]

Active ingredient

The active ingredient (CX-024414) is the mRNA encoding for the pre-fusion-stabilized Spike (S) protein of SARS-Cov-2. The S protein consists of two subunits (S1 and S2) and is stabilized in the so-called pre-fusion conformation by two amino acid mutations, K986P and V987P.
The mRNA sequence includes a 5'-cap, the 5'-UTR untranslated region, the Open Reading Frame (ORF), the 3'-UTR and the 3'-polyA tail.
RNA contains modified N1-methylpseudouridine instead of uridine to minimize indiscriminate recognition of mRNA by pathogen-associated molecular pattern receptors (e.g., TLRs).
The Figure below illustrates the general structure of the RNA encoding for the antigen.

The CX-024414 mRNA production process involves several major steps.
Capless mRNA is transcribed from linear DNA using an in vitro transcription reaction (IVT) followed by purification and filtration steps.
Next, the mRNA is enzymatically blocked followed by further purification and filtration steps. Finally, CX-214414 mRNA is filtered, packaged and stored.

The assessment report provides no other information regarding the sequence of the construct and its production industry, just as it does not provide sufficient data on safety studies of LNP's new excipients.

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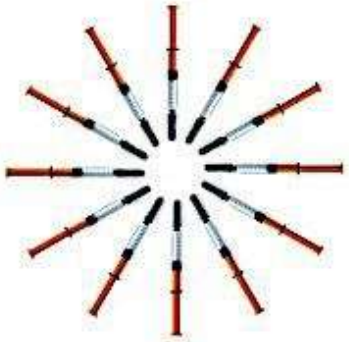
HOW ARE RNA VACCINES MADE?

RNA vaccines produced by Pfizer and BioNTech and Moderna have become the first COVID-19 vaccines approved for emergency use in the US. How are these vaccines made?



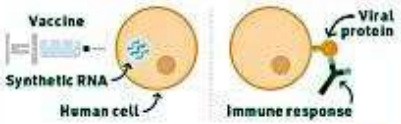
WHAT ARE RNA VACCINES?

SARS-CoV-2, the virus that causes COVID-19, uses RNA as its genetic material. Just like DNA, RNA is made up of nucleotides.

SARS-CoV-2 RNA
29,811 nucleotides long

RNA VACCINES
Moderna: 3,819 nucleotides long
Pfizer and BioNTech: 4,284 nucleotides long

RNA vaccines deliver synthetic RNA that codes for a viral protein. Our cells take up the RNA and synthesize the protein, which then generates an immune response.

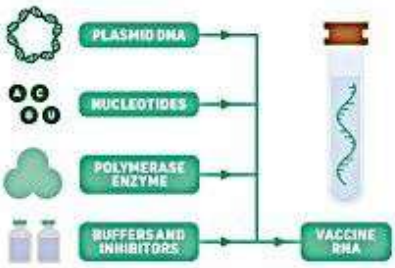


RNA PRODUCTION

Manufacturers first make a plasmid DNA template to produce the synthetic RNA. They use bacterial cells to churn out large amounts of the template.

1. **DNA OLIGONUCLEOTIDES** — Synthetic strands that are 50–100 nucleotides long
2. **PLASMID DNA TEMPLATE** — Formed by linking strands with DNA ligation enzymes
3. **DNA AMPLIFICATION** — DNA plasmids scaled up in bacterial cells

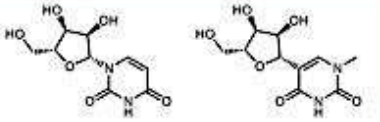
A transcription reaction that is run with enzymes and RNA nucleotides produces the RNA from the DNA template.



Finally, manufacturers purify the RNA for use in the vaccine.

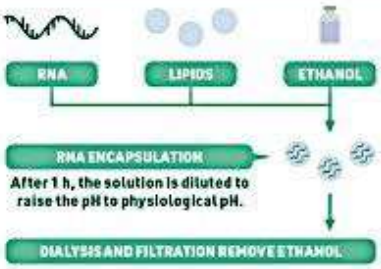
FROM RNA TO VACCINE

A key part of the RNA synthesis is the use of modified nucleotides. These modified nucleotides enhance RNA stability and prevent our immune system from breaking down the nucleic acids.



Uridine — Original nucleotide
N¹-Methylpseudouridine — Modified nucleotide

If the vaccine contained the RNA alone, enzymes would quickly destroy the nucleic acids before they could enter our cells. Encapsulating the RNA in lipid nanoparticles helps protect the RNA.



Several different lipids make up the nanoparticles. Some lipids help the particles form; others aid the structure or stability of the nanoparticle wall.

PERIODIC GRAPHICS

Production according to Good Manufacturing Practices (GMP)

All the enzymes and reaction components required for GMP production of mRNA can be obtained from commercial suppliers, such as synthetic chemicals or reagents expressed from bacteria without animal components, thus avoiding the adventitious agent-related safety issues that plague cell culture-based vaccine production.
All components, such as plasmid DNA, phage polymerases, capping enzymes, and NTPs, are readily available as GMP-grade traceable components; however, some of these are currently available only on a limited or high-cost scale.

GMP production of mRNA begins with DNA template production and enzymatic IVT and follows the same multistep protocol used for research-scale synthesis, with added controls to ensure the safety and potency of the product[153] .
To initiate the production process, the plasmid DNA template produced in Escherichia coli is linearized using a restriction enzyme that enables the synthesis of transcripts with a poly (A) tract at the 3' end. Next, the mRNA is synthesized using a bacteriophage DNA-dependent RNA polymerase (such as T7, SP6 or T3).
The template DNA is then degraded by incubation with DNase, and finally, the mRNA is blocked with enzymes or chemically to allow efficient translation in vivo. The mRNA synthesis is highly productive, leading to more than 2 g/L of full-length mRNA under optimized conditions.[154]
As pharmaceuticals, mRNA vaccines must meet regulatory purity standards. Purification of mRNA transcripts is a crucial step, and therefore a standard procedure is required in the production of mRNA vaccines to achieve efficient protein expression and adequate immunogenicity.
Some results have shown that mRNA purification can lead to a 1,000-fold increase in protein production in human primary DCs. [155]

Then, once synthesized, the mRNA goes through several purification steps to remove components of the reaction, including enzymes, free nucleotides, residual DNA and truncated RNA fragments.

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J Adv Manuf Process, (2020) 2: e10060. https://doi.org/10.1002/amp2.10060
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While precipitation with LiCl is routinely used for laboratory-scale preparation, clinical-scale purification uses batch or column derivatized microspheres, which are easier to use on a large scale[156] .
For some mRNA platforms, removal of dsRNA and other contaminants is critical to the potency of the final product, since as already seen it is a potent inducer of interferon-dependent translation inhibition.
Purification by HPLC removes IVT-derived dsRNA and thus increases protein expression.
However, reverse-phase HPLC purification coupled with ion exchange has some disadvantages such as high cost of equipment and consumables, difficulty in cost-effectively upgrading the process, handling of hazardous acetonitrile waste, loss of mRNA yield during purification (50 percent recovery) and long purification time.[157]

Recently, a new purification method[158] has been described that could overcome the major disadvantages of HPLC by providing comparable purification from dsRNA.
This method uses selective binding of dsRNA to a cellulose powder with an ethanol-containing buffer and allows up to 90 percent elimination of dsRNA. Cellulose can be used in a rotating column without the need for expensive equipment.
The method is inexpensive, fast and scalable, being suitable for large-scale purification of IVT-mRNA using rapid protein liquid chromatography (FPLC), without toxic or hazardous waste generated in the process.
Finally, after intravenous injection into mice, comparable antigen expression is observed between cellulose-purified mRNA and by HPLC, while the lowest expression is shown for unpurified mRNA.
After the mRNA has been purified, it is added to a final storage buffer and filtered under sterile conditions for subsequent filling into vials for clinical use.

RNA is susceptible to degradation by both enzymatic and chemical means, and therefore formulation buffers must be tested to ensure that they are free of contaminating RNases and may contain buffer components, such as antioxidants and chelators, that minimize the effects of reactive oxygen species and bivalent metal ions that lead to mRNA instability[159] .
Pharmaceutical formulation of mRNAs is an active area of development. Although most products for early phase studies are stored frozen (-70°C), efforts continue to develop stable formulations at higher temperatures more suitable for vaccine delivery.
Published reports suggest that stable formulations can be made at room or refrigerated temperatures.
It has been reported that the RNActive platform is active after freeze-drying and storage at 5-25°C for 3 years and at 40°C for 6 months[160] .

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Critical considerations for developing nucleic acid macromolecule-based drug products.
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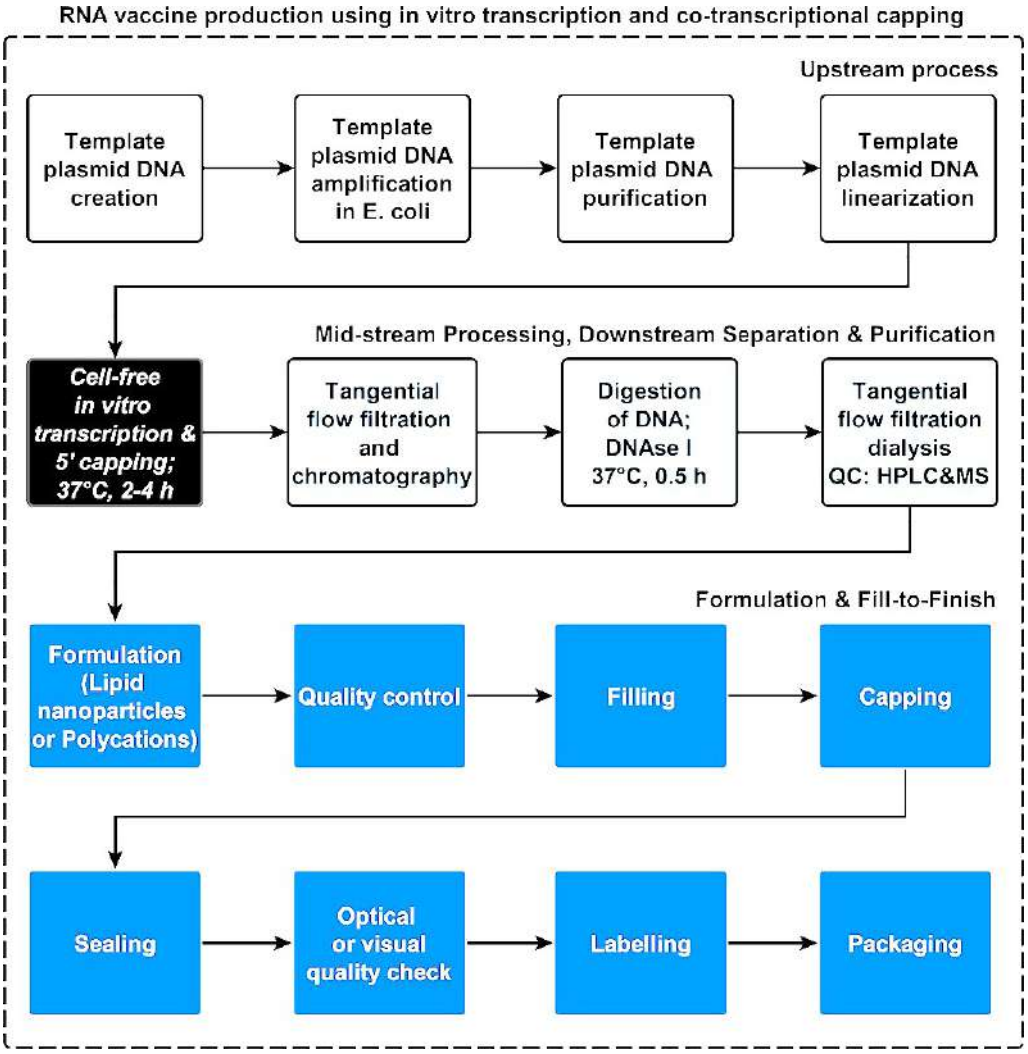
Safety and immunogenicity of a rabies vaccine mRNA in healthy adults: an open-label, nonrandomized, prospective, first-in-human phase 1 clinical trial.

Another report showed that freeze-dried naked mRNA is stable for at least 10 months under refrigerated conditions [161].

The stability of mRNA products could also be improved by encapsulating it within nanoparticles or by co-formulation with RNase inhibitors[162] .



https://aiche.onlinelibrary.wiley.com/doi/full/10.1002/amp2.10060
Flowchart of the process for mRNA vaccine production based on the enzymatic transcription reaction in vitro. In the upstream process, template DNA is generated, amplified, purified and linearized. In the mid - stream process, RNA is synthesized following the in vitro transcription reaction using the enzyme T7 RNA polymerase, and 5 ′ capping of the RNA is co-transcriptionally achieved using 5′-cap analogs (required to ensure antigen expression). For downstream purification, TFF can also be used in combination with chromatographic methods, such as hydroxyapatite chromatography and flow-through sphere chromatography. In the first TFF step, mRNA and the linearized DNA template are retained by the filter and smaller molecular size components, including the T7 RNA polymerase enzyme, flow through the filter. Next, the linearized DNA template is digested using nuclease, and then the DNA nucleotides can be separated from the RNA using another TFF step. The resulting drug substance is then formulated predominantly into lipid nanoparticles, however, polycationic formulations are also developed and evaluated. Next, the formulated mRNA undergoes quality control and is placed in vials or containers for pandemic-scale mass vaccination. The vials are then capped, sealed, inspected using automated image processing, labeled, and packaged in secondary and tertiary packaging. The entire production process is

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1524753/

independent of RNA sequence, so in principle vaccines against virtually any disease can be produced using the same manufacturing process [163]

mRNA Vehiculation

The delivery system is a key element in mRNA vaccines, as it should protect the mRNA from RNases and enhance delivery into the cytosol.
For efficient stimulation of the innate immune system, it should be able to promote mRNA release in immune cells including APCs[164] , B cells[165] or T cells[166] .
The release of mRNA molecules into cells is challenging, as an mRNA molecule is 3-4 times larger than any molecule that can spontaneously diffuse across the cytoplasmic membrane, and in addition, mRNA molecules are negatively charged and thus are rejected by the cytoplasmic membrane.

There are two basic approaches for vaccine mRNA delivery (therapeutic and prophylactic use) described to date. The first used is introduction of mRNA into DCs (dendritic cells) *ex vivo*, followed by reinfusion of the transfected cells[167] ; and the second is direct parenteral injection of mRNA with or without a vehicle. [168]
The use of *ex vivo* transfected DCs allows precise control of cell targeting, transfection efficiency, and other cellular conditions, but as a form of cell therapy, it is an expensive and laborious approach to vaccination. Direct mRNA injection is relatively rapid and inexpensive, but still does not allow for specific and efficient delivery into the target cell type, although there have been recent advances in this regard.[169]

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Numerous platforms for mRNA delivery have been described, and some have already been tested in clinical trials[170] prior to the COVID-19 prophylaxis trial, which include simple transporters, such as protamine[171] , dendrimers[172] , polyethylenimine[173] or more complex colloidal complexes such as nanoparticle and lipid mixtures[174] or nanoparticles and peptides that penetrate cells[175] .

Main mRNA vaccine delivery methods

Commonly used delivery methods and carrier molecules for mRNA vaccines are shown along with typical diameters for particle complexes:
Naked mRNA (part a);
naked mRNA with in vivo electroporation (part b);
protamine (cationic peptide)-complex mRNA (part c);
mRNA associated with a positively charged oil-in-water cationic nanoemulsion (part d);
mRNA associated with a chemically modified dendrimere and complexed with polyethylene glycol (PEG)-lipid (part e);
mRNA complexed with protamine in a PEG lipid nanoparticle (part f);
mRNA associated with a cationic polymer such as polyethylenimine (PEI) (part g);

Systemic RNA delivery to dendritic cells exploits antiviral defense for cancer immunotherapy. Nature. 2016 Jun 16;534(7607):396-401. doi: 10.1038/nature18300. Epub 2016 Jun 1.
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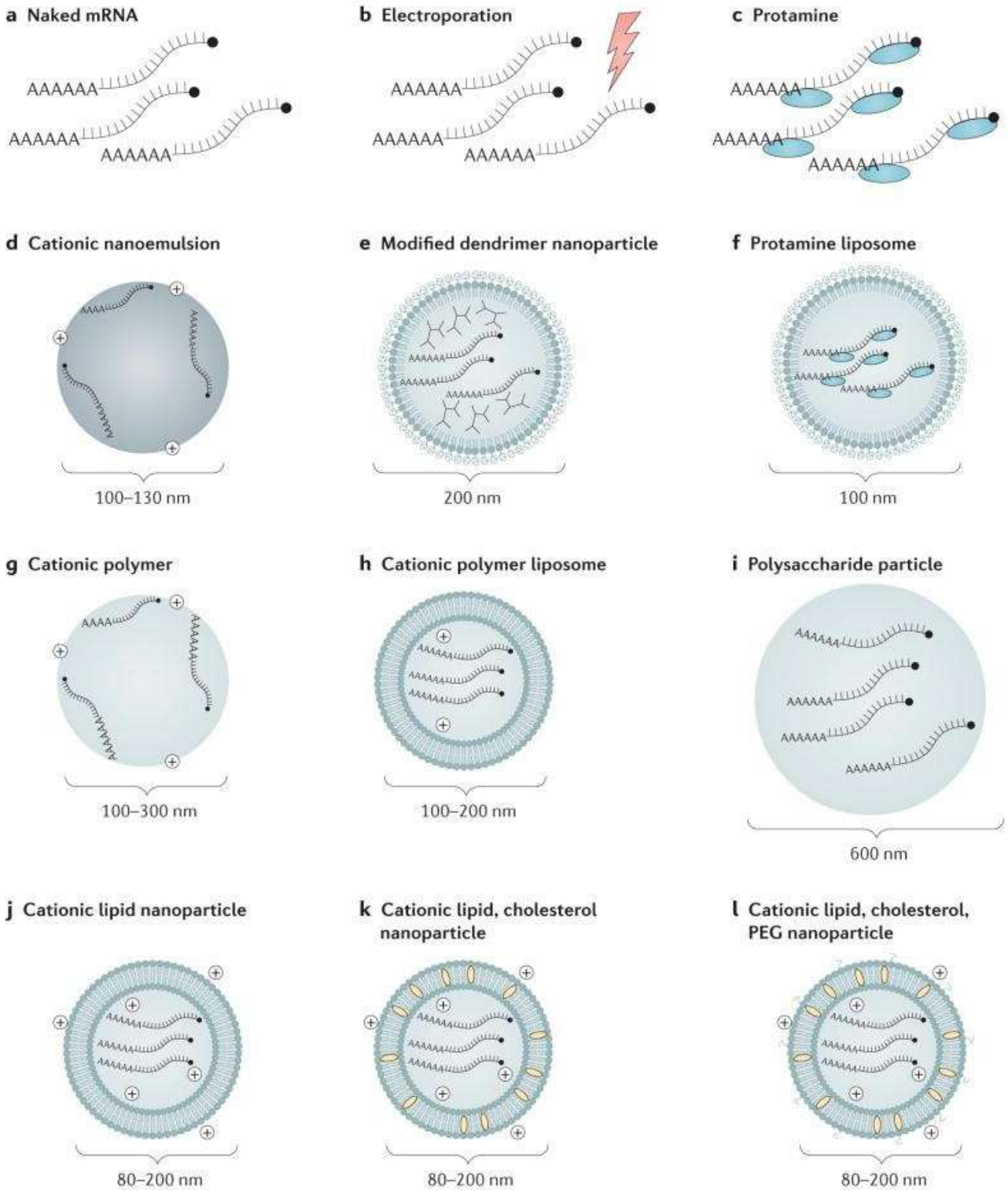
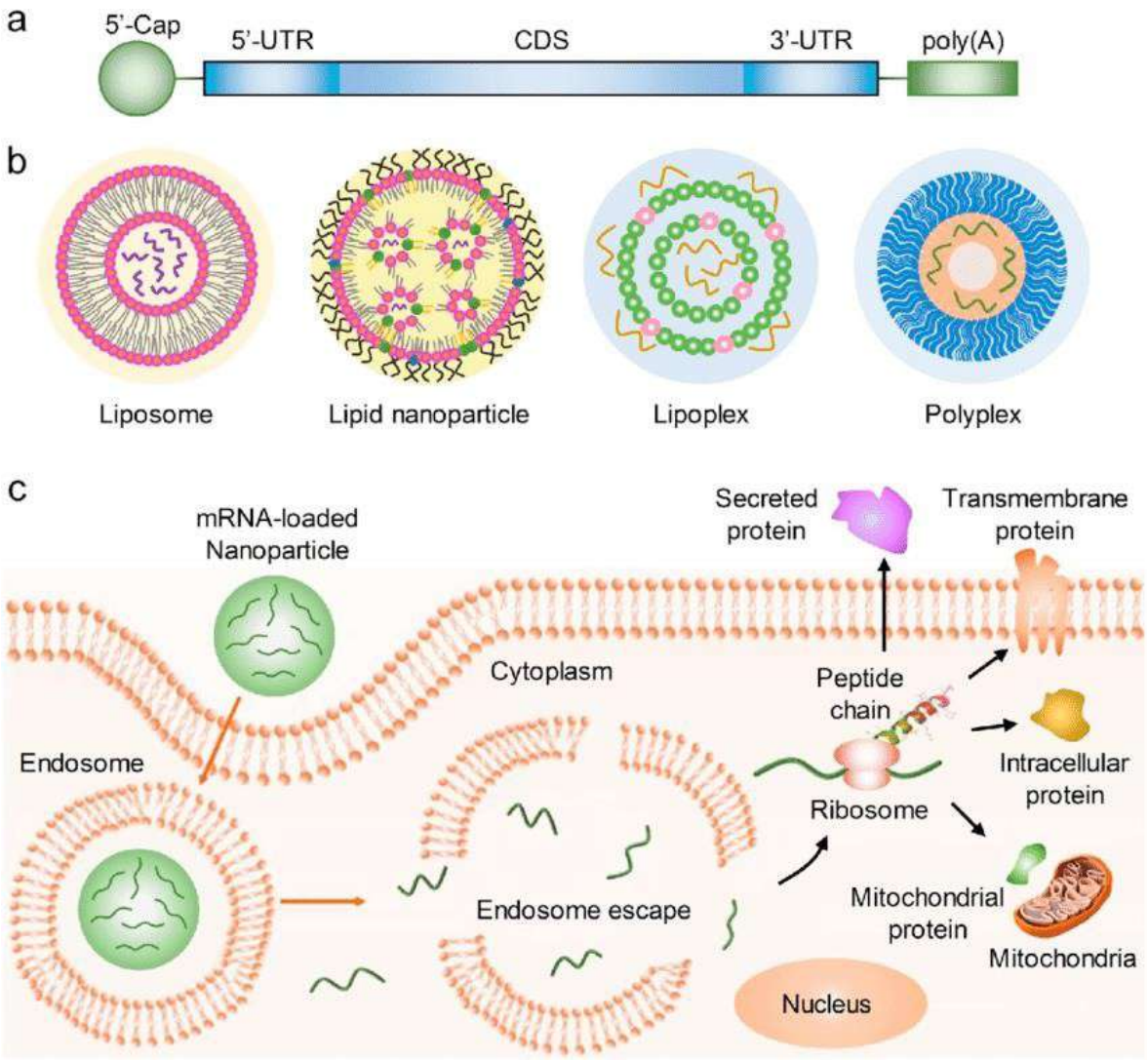
Immunogenicity Testing of Lipidoids In Vitro and In Silico: Modulating Lipidoid-Mediated TLR4 Activation by Nanoparticle Design.
Mol Ther Nucleic Acids. 2018 Jun 1;11:159-169. doi: 10.1016/j.omtn.2018.02.003. Epub 2018 Feb 13.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5992342/

[175] Coolen AL, Lacroix C, Mercier-Gouy P, Delaune E, Monge C, Exposito JY, Verrier B.
Poly(lactic acid) nanoparticles and cell-penetrating peptide potentiate mRNA-based vaccine expression in dendritic cells by triggering their activation.
Biomaterials. 2019 Mar;195:23-37. doi: 10.1016/j.biomaterials.2018.12.019. Epub 2018 Dec 21.
https://pubmed.ncbi.nlm.nih.gov/30610991/

mRNA associated with a cationic polymer such as PEI and a lipid component (part h); mRNA associated with a polysaccharide particle or gel (e.g., chitosan) (part i);
mRNA in a cationic lipid nanoparticle (e.g., lipid 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) or dioleoylphosphatidylethanolamine (DOPE)) (part j);
mRNA complexed with cationic lipids and cholesterol (part k); and mRNA complexed with cationic lipids, cholesterol, and PEG-lipids (part l)



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5906799/



https://www.researchgate.net/figure/mRNA-delivery-and-protein-expression-a-Scheme-of-the-structure-of-in-vitro-transcribed_fig2_339409095
Vehiculation of mRNA and protein expression. (a) Schematic diagram of the structure of mRNA transcribed in vitro (IVT). (b) Representative mRNA delivery formulations. (c) Cellular uptake of mRNA and protein expression process.

In the case of COVID-19 vaccines, mainly lipid nanoparticles (LNPs) are used as mRNA vehicles.
LNP complexes are the most widely used platform and present the best results in mRNA delivery.

LNPs are composed mainly of ionizable lipids, cholesterol, phospholipids, and lipid-anchored polyethylene glycol (PEG), and in addition to their role in mRNA protection, they facilitate cellular uptake, enhance exit from endosomes, and allow release into the cytoplasm.
LNPs can also protect mRNA molecules from recognition in endosomes by TLRs, preventing excessive activation of the innate immune system.
However, there remain some critical issues that need to be overcome in the administration of mRNA vaccines,[176] such as:

- The toxicity of some lipid formulations;

[176] Kowalski PS, Rudra A, Miao L, Anderson DG.
Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery.
Mol Ther. 2019 Apr 10;27(4):710-728. doi: 10.1016/j.ymthe.2019.02.012. Epub 2019 Feb 19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453548/

Granot Y, Peer D.
Delivering the right message: Challenges and opportunities in lipid nanoparticles-mediated modified mRNA therapeutics-An innate immune system standpoint.
Semin Immunol. 2017 Dec;34:68-77. doi: 10.1016/j.smim.2017.08.015. Epub 2017 Sep 7.
https://pubmed.ncbi.nlm.nih.gov/28890238/

- The difficulty in reaching immune cells in dedicated secondary lymphoid organs;
- The need to adapt each delivery system to the route of administration.

To overcome these drawbacks, transport and egress from endosomes of mRNA-LNP vehicles can be improved[177] using, for example, different ionizable lipids that promote strong cellular and humoral immune responses.
Such ionizable lipids are neutral or slightly charged at physiological pH 7.4 and thus have a good safety profile. More recently, new branched-tail LNPs have been designed to overcome the mRNA size-dependent release limitation[178] .
In particular, the ionization of these nanocarriers at pH 5.0 (the late endosomal pH) correlates with the translation efficiency of the transported mRNA, and it appears that such colloids could be a vehicle for mRNA of any size.
This emphasizes the relevance of chemical interactions between mRNA and formulation components for mRNA expression in vivo.

Insight Materials used for the delivery of nonviral mRNA [179]

Among the many barriers to function, mRNA must cross the cell membrane to reach the cytoplasm. The cell membrane is a dynamic and formidable barrier to intracellular release.
It consists mainly of a lipid bilayer of zwitterionic and negatively charged phospholipids, where the polar heads of the phospholipids point toward the aqueous environment and the hydrophobic tails form a hydrophobic core.[180]

Various ion pumps and ion channels help maintain a negative potential (-40 to -80 mV) across the cell membrane and keep the cytoplasmic space negatively charged by controlling the balance of most essential metal ions (e.g., K^+ , Na^+ , Ca^{2+} and Mg^{2+}) .[181]
The negative potential across the cell membrane creates an insurmountable barrier for mRNA molecules that are strongly negatively charged.

Unsaturated lipids, particularly *cis* double-bonded lipids, increase the fluidity of the cell membrane, while the other major components of the bilayer include sterols (~30% of total lipids), of which Cholesterol is the major sterol, which helps maintain a balance between fluidization and condensation of the bilayer
lipid.

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[177] Kauffman KJ, Dorkin JR, Yang JH, Heartlein MW, DeRosa F, Mir FF, Fenton OS, Anderson DG. Optimization of Lipid Nanoparticle Formulations for mRNA Delivery in Vivo with Fractional Factorial and Definitive Screening Designs.
Nano Lett. 2015 Nov 11;15(11):7300-6. doi: 10.1021/acs.nanolett.5b02497. Epub 2015 Oct 20.
https://pubmed.ncbi.nlm.nih.gov/26469188/

Hassett KJ, et al Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines.
Mol Ther Nucleic Acids. 2019 Apr 15;15:1-11. doi: 10.1016/j.omtn.2019.01.013. Epub 2019 Feb 7.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6383180/

[178] Hajj KA, Ball RL, Deluty SB, Singh SR, Strelkova D, Knapp CM, Whitehead KA.
Branched-Tail Lipid Nanoparticles Potently Deliver mRNA In Vivo Due to Enhanced Ionization at Endosomal pH.
Small. 2019 Feb;15(6):e1805097. doi: 10.1002/smll.201805097. Epub 2019 Jan 13.
https://pubmed.ncbi.nlm.nih.gov/30637934/

[179] Kowalski PS, Rudra A, Miao L, Anderson DG.
Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery.
Mol Ther. 2019 Apr 10;27(4):710-728. doi: 10.1016/j.ymthe.2019.02.012. Epub 2019 Feb 19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453548/

[180] Harayama T, Riezman H.
Understanding the diversity of membrane lipid composition.
Nat Rev Mol Cell Biol. 2018 May;19(5):281-296. doi: 10.1038/nrm.2017.138. Epub 2018 Feb 7. Erratum in: Nat Rev Mol Cell Biol. 2019 Nov;20(11):715.
https://pubmed.ncbi.nlm.nih.gov/29410529/.

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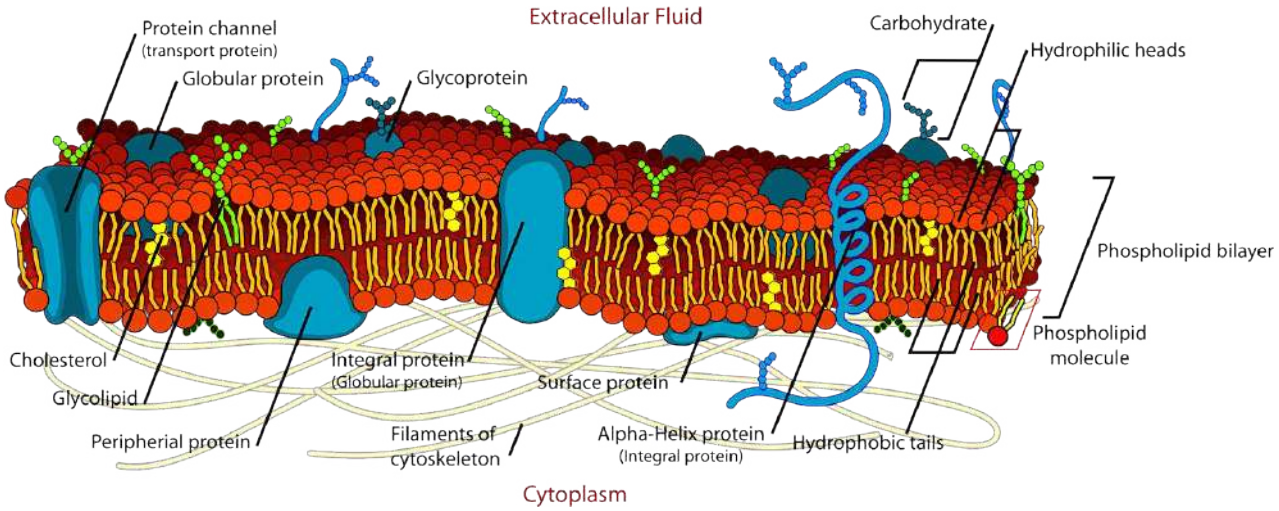
Electrostatic interactions in membranes and proteins.
Annu Rev Biophys Biophys Chem. 1986;15:163-93. doi: 10.1146/annurev.bb.15.060186.001115.
https://pubmed.ncbi.nlm.nih.gov/2424473/



https://opm.phar.umich.edu/biological_membranes

In addition to the cell membrane barrier, mRNA faces degradation by extracellular ribonucleases found in abundance in the skin and blood. [182]

To protect mRNA from degradation by nucleases and shield its negative charge, amine-containing materials are commonly used as nonviral carriers. One of the most developed methods for mRNA transport is, as already seen, co-formulation into lipid nanoparticles (LNPs)[183] .

LNP formulations are typically composed of.

(1) an ionizable or cationic lipid or polymeric material containing tertiary or quaternary amines to encapsulate the polyanionic mRNA;
(2) a zwitterionic lipid (e.g., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine [DOPE]) that resembles lipids in the cell membrane;
(3) Cholesterol to stabilize the lipid bilayer of the LNP; and
(4) a polyethylene glycol (PEG)-lipid to give the nanoparticle a moisturizing layer, improve colloidal stability, and reduce protein absorption.[184]

Although the mechanism of mRNA release by LNPs is not completely understood, it is generally accepted that these multicomponent LNPs are taken up by endocytosis and can bind through electrostatic bonds and fuse with the cell membrane. [185]

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[182] Houseley J, Tollervey D.
The many pathways of RNA degradation.
Cell. 2009 Feb 20;136(4):763-76. doi: 10.1016/j.cell.2009.01.019. P
https://www.cell.com/cell/fulltext/S0092-8674(09)00067-1

Tsui NB, Ng EK, Lo YM.
Stability of endogenous and added RNA in blood specimens, serum, and plasma.
Clin Chem. 2002 Oct;48(10):1647-53. PMID: 12324479.
https://pubmed.ncbi.nlm.nih.gov/12324479/

[183] Kauffman KJ, Dorkin JR, Yang JH, Heartlein MW, DeRosa F, Mir FF, Fenton OS, Anderson DG.
Optimization of Lipid Nanoparticle Formulations for mRNA Delivery in Vivo with Fractional Factorial and Definitive Screening Designs.
Nano Lett. 2015 Nov 11;15(11):7300-6. doi: 10.1021/acs.nanolett.5b02497. Epub 2015 Oct 20.
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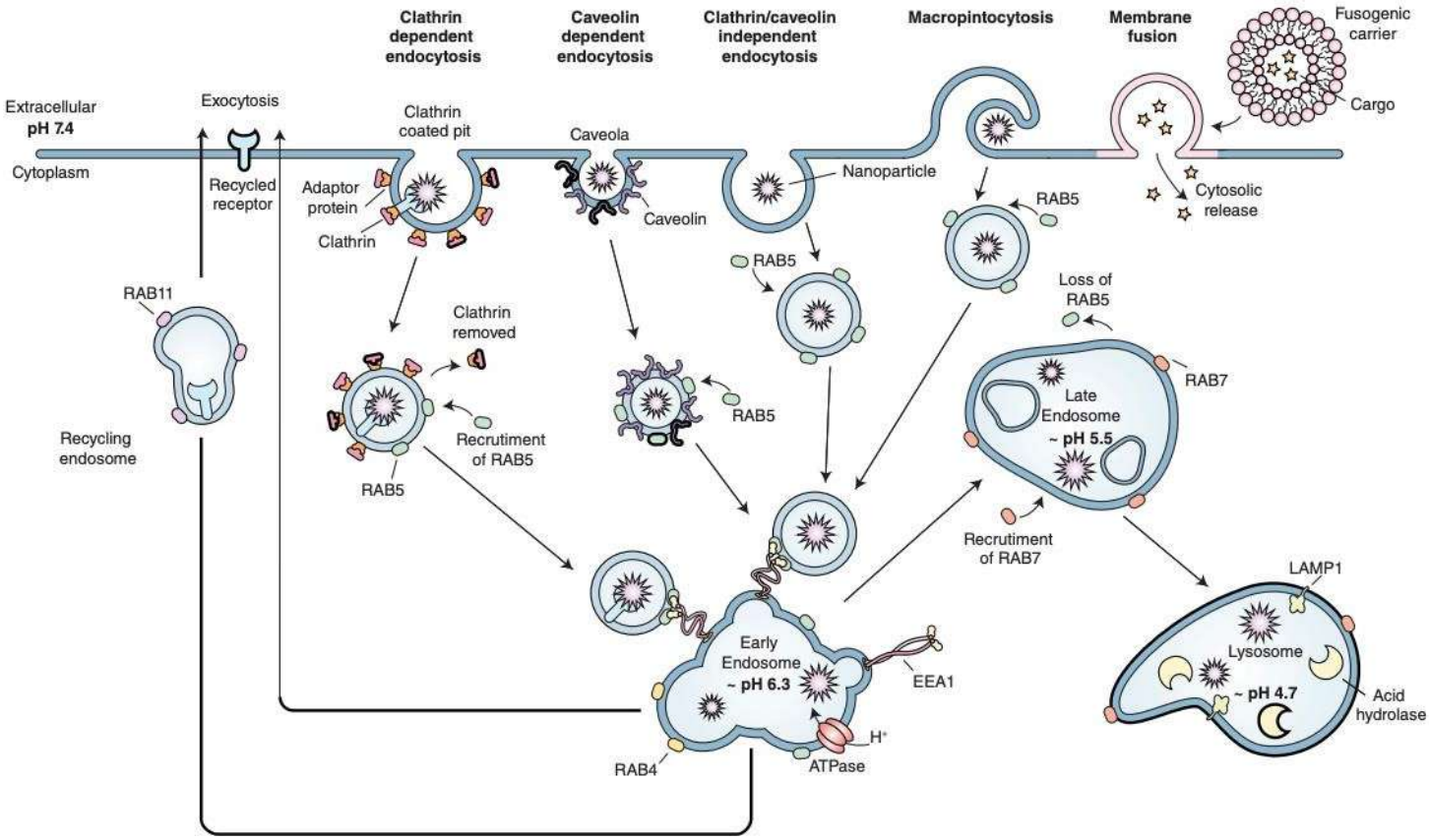
Principles of nanoparticle design for overcoming biological barriers to drug delivery.
Nat Biotechnol. 2015;33(9):941-951. doi:10.1038/nbt.3330
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4978509/

Semple SC,
Rational design of cationic lipids for siRNA delivery.
Nat Biotechnol. 2010 Feb;28(2):172-6. doi: 10.1038/nbt.1602. Epub 2010 Jan 17.
https://pubmed.ncbi.nlm.nih.gov/20081866/

[185] Yanez Arteta M, Kjellman T, Bartesaghi S, et al.

It is important to keep in mind that LNPs can also be exocytosed, hindering cellular release.[186] Initial clathrin-dependent endocytosis and macropinocytosis, have been identified as common mechanisms for LNP release within cells. [187]

Once inside the cell, LNPs are directed into early endosomes, then into late endosomes, and finally into lysosomes where the mRNA content is degraded enzymatically. [188]



https://pubmed.ncbi.nlm.nih.gov/28160452/
Nanoparticles can enter cells through multiple mechanisms. Particles are absorbed into vesicles coated with clathrin, caveolin, or using a clathrin/caveolin-independent mechanism. EEA1 binds to RAB5 on internalized vesicles and attracts them into early endosomes positive for

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Successful reprogramming of cellular protein production through mRNA delivered by functionalized lipid nanoparticles.
Proc Natl Acad Sci U S A. 2018;115(15):E3351-E3360. doi:10.1073/pnas.1720542115
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5899464/

[186] Sahay G, Querbes W, Alabi C, et al.
Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling.
Nat Biotechnol. 2013;31(7):653-658. doi:10.1038/nbt.2614
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3814166/

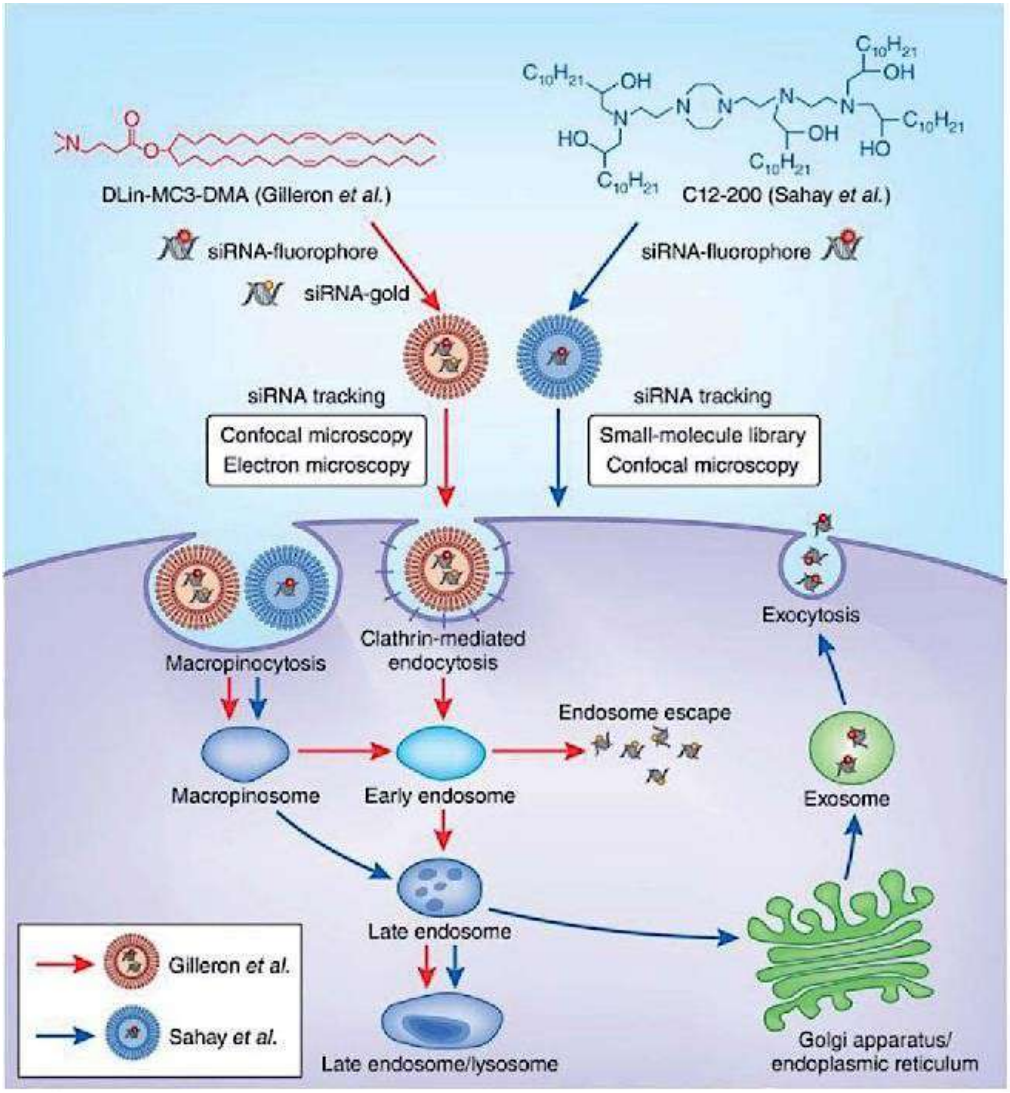
[187] Gilleron J, et al
Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape.
Nat Biotechnol. 2013 Jul;31(7):638-46. doi: 10.1038/nbt.2612. Epub 2013 Jun 23.
https://pubmed.ncbi.nlm.nih.gov/23792630/

But D.
Enhancing endosomal escape for nanoparticle-mediated siRNA delivery.
Nanoscale. 2014 Jun 21;6(12):6415-25. doi: 10.1039/c4nr00018h.
https://pubmed.ncbi.nlm.nih.gov/24837409/

Dominska M, Dykxhoorn DM.
Breaking down the barriers: siRNA delivery and endosome escape.
J Cell Sci. 2010 Apr 15;123(Pt 8):1183-9. doi: 10.1242/jcs.066399.
http://jcs.biologists.org/cgi/pmidlookup?view=long&pmid=20356929

[188] ur Rehman Z, Hoekstra D, Zuhorn IS.
Mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis.
ACS Nano. 2013 May 28;7(5):3767-77. doi: 10.1021/nn3049494. Epub 2013 Apr 24.
https://pubmed.ncbi.nlm.nih.gov/23597090/

RAB5. The pH drops to ~6.3 and the cargo is recycled to the surface or transferred to RAB7-positive late endosomes (pH ~5.5). Contents are transferred to the lysosome (pH ~ 4.7) where they are degraded by acid hydrolases. Alternative entry modes include membrane fusion or direct translocation across the membrane, bypassing the trafficking pathway.



https://www.semanticscholar.org/paper/Enhancing-endosomal-escape-for-nanoparticle-siRNA-Ma/d92abec09beec91102a82479e6233f533c17077a

A hypothesis called the "proton sponge effect" proposes that a small percentage (1% -2%) of LNPs escape degradation because the gradual ATP-driven acidification from pH 6.5 to 5-6 of the compartments promotes protonation of the residual amines of LNPs and destroys the endosomal membrane leading to the escape from the endosomes of mRNA. [189]

Other studies, however, indicate that the actual mechanism may be much more complex.[190]

[189] Martens, Thomas, K. Remaut, J. Demeester, S. Smedt and K. Braeckmans.
Intracellular delivery of nanomaterials: how to catch endosomal escape in the act. Nano Today 9 (2014): 344-364.
https://doi.org/10.1016/j.nantod.2014.04.011
https://biblio.ugent.be/publication/5760110/file/5760123.pdf

Selby LI, Cortez-Jugo CM, Such GK, Johnston APR.
Nanoescapology: progress toward understanding the endosomal escape of polymeric nanoparticles.
Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2017 Sep;9(5). doi: 10.1002/wnan.1452. Epub 2017 Feb 3.
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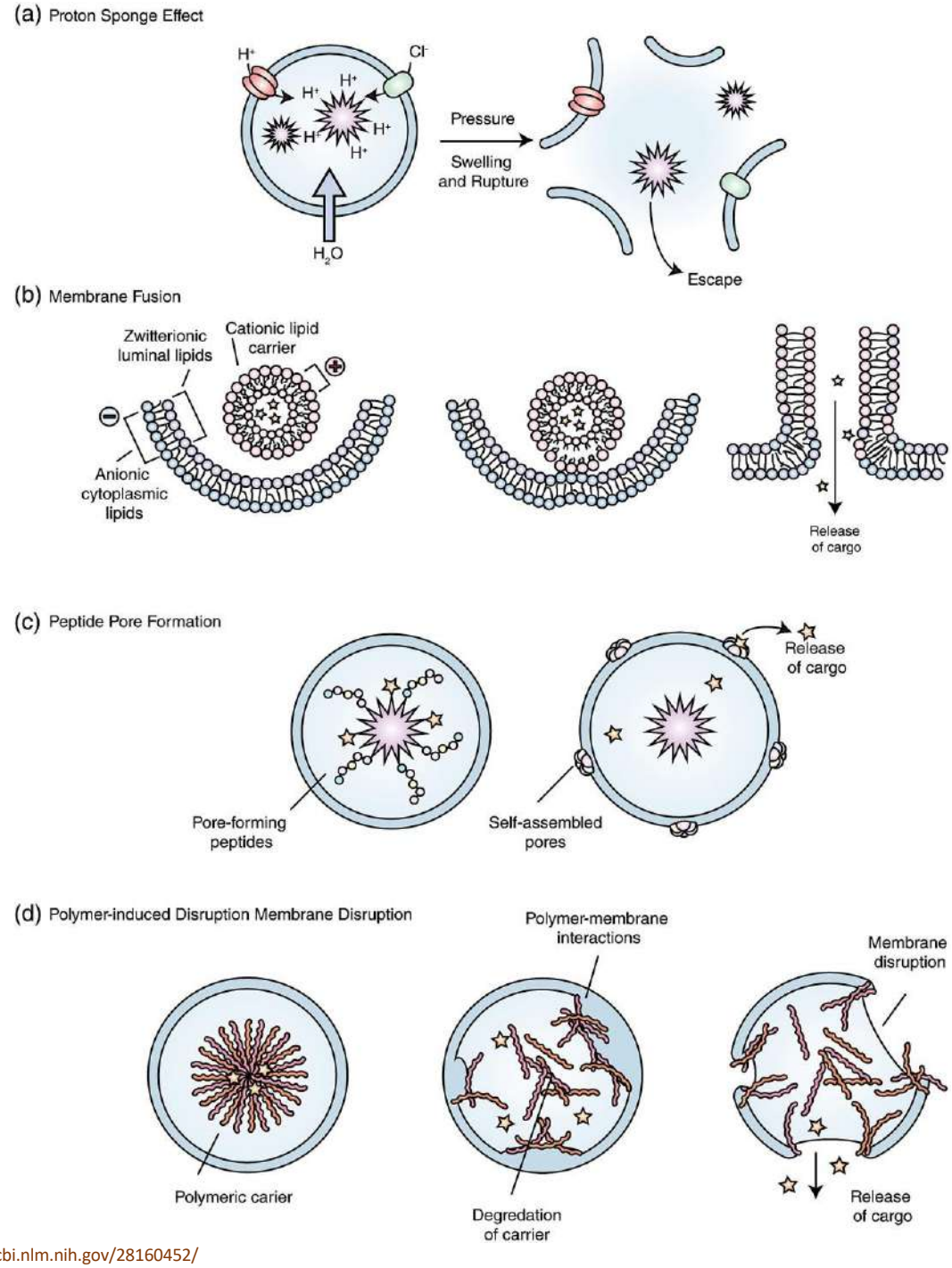
[190] Vermeulen LMP, Brans T, Samal SK, Dubruel P, Demeester J, De Smedt SC, Remaut K, Braeckmans K.
Endosomal Size and Membrane Leakiness Influence Proton Sponge-Based Rupture of Endosomal Vesicles.
ACS Nano. 2018 Mar 27;12(3):2332-2345. doi: 10.1021/acsnano.7b07583. Epub 2018 Mar 9.
https://pubmed.ncbi.nlm.nih.gov/29505236/

Li B, Zhang X, Dong Y.
Nanoscale platforms for messenger RNA delivery.
Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2019;11(2):e1530. doi:10.1002/wnan.1530
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6443240/



https://pubmed.ncbi.nlm.nih.gov/28160452/
Endosomal escape mechanisms.
(a) Proton sponge effect: polymers capable of buffering become protonated when protons are pumped into endosomes as part of the regular ATPase trafficking process. Chloride ions are also transported to maintain charge balance within the endosome. Increased ion concentration causes osmotic swelling and membrane rupture.[191]
(b) Membrane fusion: anionic lipids on the cytoplasmic side of endosomes reorganize to form a neutral ion pair with cationic transporter lipids, destabilizing the membrane.

Alfagih IM, Aldosari B, AlQuadeib B, Almurshedi A, Alfagih MM.
Nanoparticles as Adjuvants and Nanodelivery Systems for mRNA-Based Vaccines.
Pharmaceutics. 2020;13(1):45. Published 2020 Dec 30. doi:10.3390/pharmaceutics13010045
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7823281/

[191] Behr, Jean-Paul
The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit
CHIMIA International Journal for Chemistry, Volume 51, Numbers 1-2, January/February 1997, pp. 34-36(3)
https://www.ingentaconnect.com/content/scs/chimia/1997/00000051/F0020001/art00026?crawler=true&mimetype=application/pdf

The membranes fuse and allow the cargo to move into the cytoplasm.[192]
(c) Pore formation: some peptides self-assemble in the lipid membrane to form pores that allow leakage of low molecular weight therapeutics.
(d) Membrane rupture: polymers or peptides interact directly with the endosomal membrane causing rupture, allowing the cargo to escape.

Ionizable lipids

A well-studied class of nonviral mRNA delivery agents includes cationic or ionizable lipids and lipid-like materials. Cationic lipids carry alkylated quaternary ammonium groups and retain their cationic nature in a pH-independent manner, while ionizable lipids acquire positive charges by protonation of free amines when the pH is lowered.[193]
Recently, regulatory agencies approved the first siRNA-based drug (Patisiran [Onpattro]), which contains an ionizable lipid called Dlin-MC3-DMA (MC3). [194]

Polymers

Polymeric materials are not as clinically advanced for nucleic acid delivery as ionizable lipids, with few formulations used for therapeutic siRNA delivery. [195]
Relative to lipids, polymeric materials face additional challenges related to polydispersion and clearance or biodegradation for large molecular weight polymers .
Low molecular weight polyethylenimine (PEI) modified with fat chains was used for siRNA and mRNA delivery to reduce the toxicity of high molecular weight PEI. [196]

Dendrimers

Dendrimers are molecules that contain a series of branches extending from a central core and generally have several copies of the same functional group at the end of the branches.

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[192] Zelphati O, Szoka FC Jr.
Mechanism of oligonucleotide release from cationic liposomes.
Proc Natl Acad Sci U S A. 1996;93(21):11493-11498. doi:10.1073/pnas.93.21.11493
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC38085/pdf/pnas01525-0218.pdf

[193] Ma Z, Li J, He F, Wilson A, Pitt B, Li S.
Cationic lipids enhance siRNA-mediated interferon response in mice.
Biochem Biophys Res Commun. 2005 May 13;330(3):755-9. doi: 10.1016/j.bbrc.2005.03.041.
https://pubmed.ncbi.nlm.nih.gov/15809061/

[194] https://www.ema.europa.eu/en/documents/product-information/onpattro-epar-product-information_it.pdf

[195] Wong SC, et al
HIF2α-Targeted RNAi Therapeutic Inhibits Clear Cell Renal Cell Carcinoma.
Mol Cancer Ther. 2018 Jan;17(1):140-149. doi: 10.1158/1535-7163.MCT-17-0471. Epub 2017 Oct 27.
https://mct.aacrjournals.org/content/17/1/140.full-text.pdf

Ramot Y, Rotkopf S, Gabai RM, Zorde Khvalevsky E, Muravnik S, Marzoli GA, Domb AJ, Shemi A, Nyska A.
Preclinical Safety Evaluation in Rats of a Polymeric Matrix Containing an siRNA Drug Used as a Local and Prolonged Delivery System for Pancreatic Cancer Therapy.
Toxicol Pathol. 2016 Aug;44(6):856-65. doi: 10.1177/0192623316645860. Epub 2016 May 4.
https://pubmed.ncbi.nlm.nih.gov/27147553/

[196] Lv H, Zhang S, Wang B, Cui S, Yan J.
Toxicity of cationic lipids and cationic polymers in gene delivery.
J Control Release. 2006 Aug 10;114(1):100-9. doi: 10.1016/j.jconrel.2006.04.014. Epub 2006 May 13.
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Khan OF, Kowalski PS, Doloff JC, et al.
Endothelial siRNA delivery in nonhuman primates using ionizable low-molecular weight polymeric nanoparticles.
Sci Adv. 2018;4(6):eaar8409. Published 2018 Jun 27. doi:10.1126/sciadv.aar8409
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6021147/

Dahlman JE, Barnes C, Khan O, et al.
In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight.
Nat Nanotechnol. 2014;9(8):648-655. doi:10.1038/nnano.2014.84
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4207430/

Zhao M, Li M, Zhang Z, Gong T, Sun X.
Induction of HIV-1 gag-specific immune responses by cationic micelles mediated delivery of gag mRNA.
Drug Deliv. 2016 Sep;23(7):2596-2607. doi: 10.3109/10717544.2015.1038856. Epub 2015 May 29.
https://pubmed.ncbi.nlm.nih.gov/26024387/

Polyamidoamine (PAMAM) or polypropylenimine-based dendrimers have been extensively studied for gene delivery. [197]

PAMAM dendrimers synthesized with a modified fatty chain for siRNA delivery were subsequently used to develop a self-replicating, single-dose, adjuvant-free mRNA vaccine platform administered intramuscularly to express antigens for Ebola, H1N1 influenza, *Toxoplasma gondii* and Zika. [198] Because the repetitive units of dendrimers branch in the shape of a tree, their enzymatic biodegradation may be hindered due to steric factors, leading to toxicity resulting from the accumulation of these materials in tissues.

Peptides that penetrate cells

Cell-penetrating peptides (CPPs) have been studied for their potential as vectors for intracellular delivery of nucleic acid. [199]

Although their mechanisms of internalization are not fully understood, it is speculated that CPPs may promote the clustering of negatively charged glycosaminoglycans on the cell surface, which in turn triggers macropinocytosis and lateral diffusion or directly breaks down the lipid bilayer. [200]

Biodegradability and targeting issues with nonviral vectors

Systemically administered LNPs carrying mRNA face multiple barriers to delivery.

The mononuclear phagocytic system (MPS), especially in the liver and spleen, is a frequent destination for injected nanoparticles because of its primary role in controlling for nanosized infectious agents. [201]

The kidney filters bare mRNA or any nanoparticle with a hydrodynamic diameter of less than 5.5 nm.[202]

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[197] Abedi-Gaballu F, Dehghan G, Ghaffari M, et al.
PAMAM dendrimers as efficient drug and gene delivery nanosystems for cancer therapy.
Appl Mater Today. 2018;12:177-190. doi:10.1016/j.apmt.2018.05.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6269116/

Khan OF, Zaia EW, Yin H, et al.
Ionizable amphiphilic dendrimer-based nanomaterials with alkyl-chain-substituted amines for tunable siRNA delivery to the liver endothelium in vivo.
Angew Chem Int Ed Engl. 2014;53(52):14397-14401. doi:10.1002/anie.201408221
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4785599/

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Dendrimer-RNA nanoparticles generate protective immunity against lethal Ebola, H1N1 influenza, and Toxoplasma gondii challenges with a single dose [published correction appears in Proc Natl Acad Sci U S A. 2016 Aug 30;113(35):E5250]. Proc Natl Acad Sci U S A. 2016;113(29):E4133-E4142.
doi:10.1073/pnas.1600299113
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4961123/

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An RNA nanoparticle vaccine against Zika virus elicits antibody and CD8+ T cell responses in a mouse model.
Sci Rep. 2017;7(1):252. Published 2017 Mar 21. doi:10.1038/s41598-017-00193-w
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Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2257919/

Verdurmen WP, Brock R.
Biological responses toward cationic peptides and drug carriers.
Trends Pharmacol Sci. 2011 Feb;32(2):116-24. doi: 10.1016/j.tips.2010.11.005. Epub 2010 Dec 15.
https://pubmed.ncbi.nlm.nih.gov/21167610/

[201] Wilhelm, S., Tavares, A., Dai, Q. et al.
Analysis of nanoparticle delivery to tumors.
Nat Rev Mater 1, 16014 (2016). https://doi.org/10.1038/natrevmats.2016.14
https://www.nature.com/articles/natrevmats201614

[202] Albanese A, Tang PS, Chan WC.
The effect of nanoparticle size, shape, and surface chemistry on biological systems.
Annu Rev Biomed Eng. 2012;14:1-16. doi: 10.1146/annurev-bioeng-071811-150124. Epub 2012 Apr 18.
https://pubmed.ncbi.nlm.nih.gov/22524388/

Most LNPs are about 100 nm in diameter and large enough to prevent them from escaping from the MPS and reaching other organs of interest. [203]
The liver, which forms an important part of the MPS, has a fenestrated vasculature and contains phagocytic cells such as Kupffer cells, which retain cationic LNPs.
In addition, large cationic LNPs cannot extravasate from the capillaries found in the lungs and thus cannot be filtered by the bloodstream into the kidney.

This can lead to the accumulation of release material in the liver, lungs or other organs. [204]
Therefore, the clearance and biodegradability of the components of the delivery system is a relevant consideration to make when developing mRNA delivery materials.

Esters are the most commonly used functional group to improve the biodegradability of biomaterials, but the *in vivo* degradation of different ester bonds may depend on the overall chemistry of the molecules and formulations. [205]
For example, both LP-01 and lipid 5 were reported to be rapidly eliminated from the liver (half-life [$t_{1/2}$] ~6 h), compared with DLin-MC3-DMA ($t_{1/2>50}$ h), with comparable if not greater protein expression.

In some tissues, the presence of ester functional groups can accelerate the degradation of LNPs, potentially limiting protein expression as well.
OF-Deg-Lin for example selectively induced protein expression in the spleen, although it was able to reach liver cells,[206] so it was speculated that this might be due to the rapid degradation of LNPs by liver enzymes.

Rational design of degradable lipids or polymers based on polyesters or polycarbonates[207] could offer better control over the degradation of delivery systems.

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Particle size-dependent organ distribution of gold nanoparticles after intravenous administration.
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Reflexive Interfaces of Poly(trimethylene carbonate)-Based Polymers: Enzymatic Degradation and Selective Adsorption
Macromolecules **2007** *40* (24), 8731-8736 DOI: 10.1021/ma071030q
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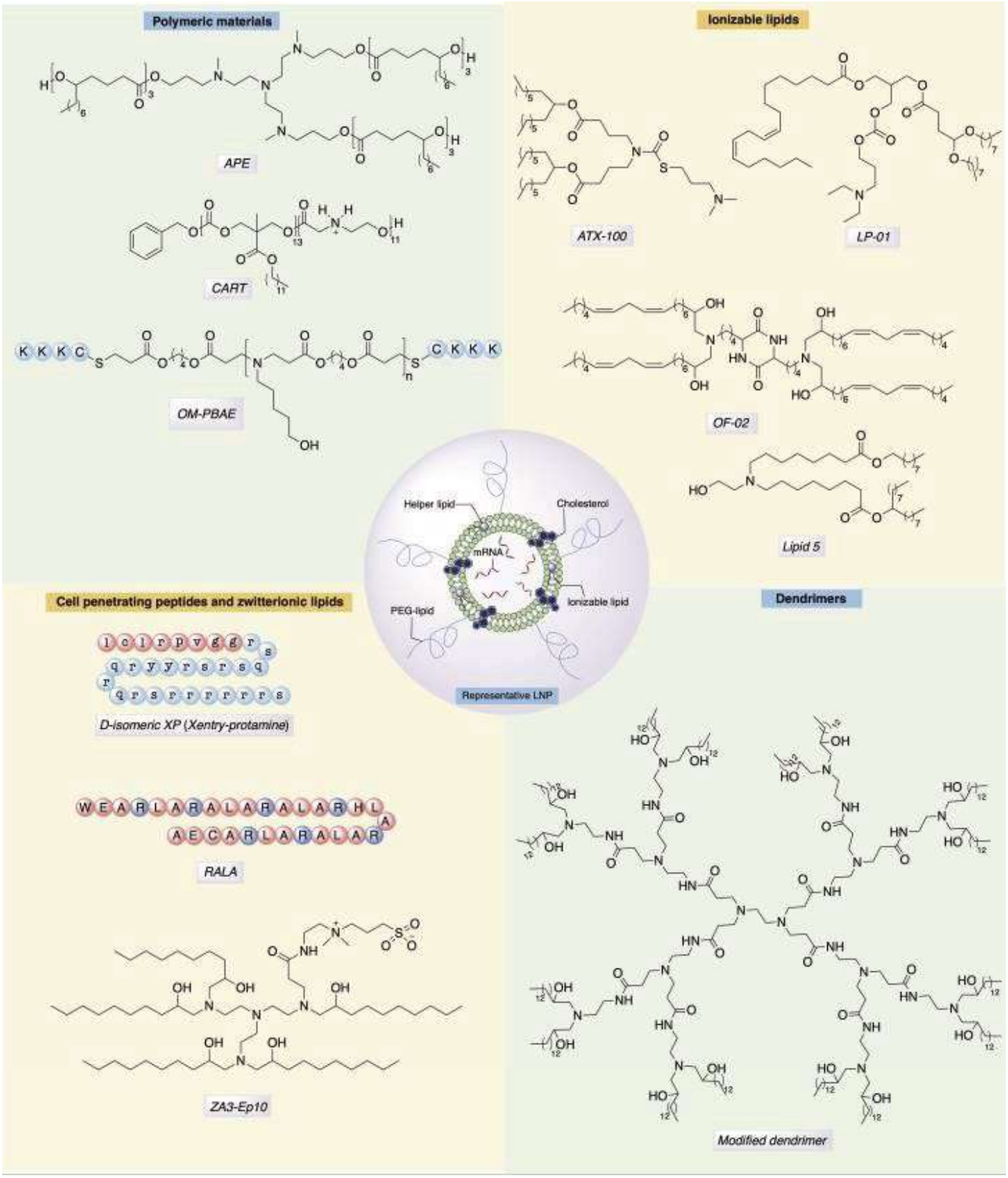


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6453548/
Representative structures of various classes of materials developed for mRNA delivery

THE PFIZER VACCINE LIPOSOME AND MODERN

Finished product of the vaccine "Pfizer"

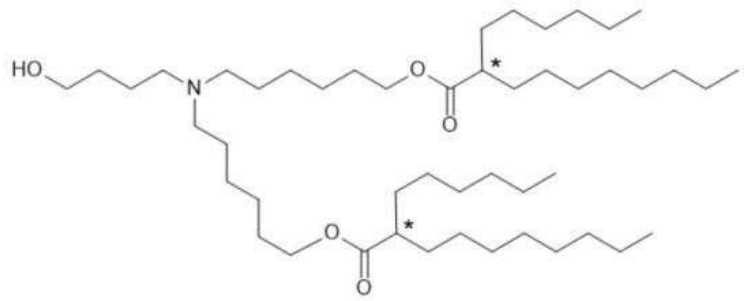
The finished product BNT162b2 is supplied as a 5-dose multidose concentrate without preservatives to be diluted before intramuscular injection. The finished product is a sterile dispersion of lipid nanoparticles (LNPs) containing RNA in an aqueous cryoprotectant buffer.

The other ingredients are:

- ALC-0315 (4-hydroxybutyl) azanediyl) bis (hexane-6,1-diyl) bis (2-hexyldecanoate),
- ALC-0159 (2 - [(polyethylene glycol)-2000]-N, N-ditetradecylacetamide), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC),
- Cholesterol,
- potassium chloride, potassium dihydrogen, phosphate, sodium chloride, disodium phosphate dihydrate, sucrose and water for injectable preparations.

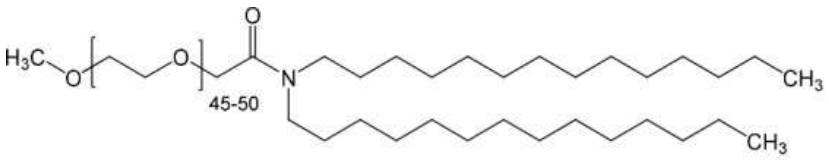
The liposome that forms the vehicle for the Pfizer vaccine contains two new excipients:
the cationic lipid ALC-0315 containing a tertiary amine and two esterified portions, and the PEGylated lipid ALC-0159 containing an acetamide functional group.

New Excipients
ALC-0315



Asterisks (*) indicate chiral centers.

ALC-0159



Both compounds are still being studied for toxicology, pharmacokinetics and pharmacodynamics because they are newly introduced.
It should be noted, however, that in the Assessment report of the Pfizer vaccine[208] it is indicated that only the whole formulation (modified RNA in LNPs) was used, so there are no toxicological data on LNP alone or its specific new excipients.
The manufacturer points out that ALC-0159 must be lost from the LNP surface to facilitate efficient uptake into target cells. At the same time, ALC-0315 is present in the LNP with a high % in moles (50% in moles) compared to the other lipids, suggesting that it is more likely that this lipid is present inside the cells (and probably in the vacuoles).
No genotoxicity or carcinogenicity studies were provided because the components of the vaccine formulation are lipids and RNAs that are not expected to have genotoxic potential.

[208] https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf

However, the new excipient ALC-0159 contains a potentially genotoxic portion of acetamide.
The risk assessment performed by the manufacturer indicates that the risk of genotoxicity related to this excipient is very low based on literature data referring to the genotoxicity of acetamide following chronic use at high doses by the oral (non-injection) route. However, this assessment is questionable because the injection and oral routes of administration are not comparable in toxicological terms.

The cationic lipid ALC-0315 due to the presence of cationic amine functional groups has the potential to trigger systemic complement activation (see below), but the manufacturer reports that although such activation is known, it has not been studied because no signs indicative of such manifestations have been detected in clinical trials. Instead, it should be noted that many adverse reactions reported in the pharmacovigilance report[209] , as discussed below, are indicative of this type of damage-inducing mechanism.

Finished product of the vaccine "Moderna"

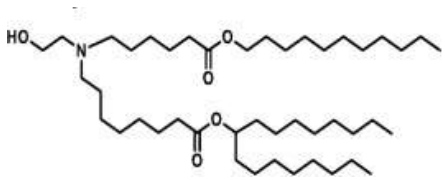
The finished product is presented as a ready-to-use white to off-white multidose dispersion for intramuscular injection.
The active ingredient is encapsulated in lipid nanoparticles (LNPs) dispersed in a diluent buffer at pH 7.5.
The LNPs are composed of four lipids that act as protectors and transporters of mRNA:

- Heptadecan-9-yl 8 - ((2-hydroxyethyl) (6-oxo-6- undecyloxy) hexyl) amino) octanoate (SM-102, a custom ionizable lipid),
- 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG2000-DMG),
- 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)
- Cholesterol.

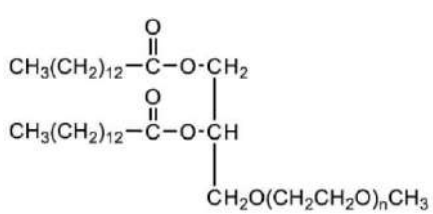
The other excipients are trometamol hydrochloride, acetic acid, sodium acetate trihydrate, sucrose, and water for injectable preparations.

SM-102 is a novel ionizable lipid excipient that is positively charged to induce lipids to interact electrostatically with mRNA when combined.
Cholesterol is incorporated to provide physicochemical stability and to the particle structure.
The zwitterionic "helper" lipid, DSPC, is incorporated to enhance the fusogenic properties of the particles.
The new polyethylene glycol-lipid conjugate excipient, PEG2000-DMG, imparts steric stabilization to the nanoparticles.
Sucrose is added to promote product stability upon freezing/thawing and for long-term storage.

New excipients
SM-102 heptadecan-9-yl 8 - ((2-hydroxyethyl) (6-oxo-6- undecyloxy) hexyl) amino) octanoate



PEG2000-DMG



[209] https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/962405/COVID-19_mRNA_Pfizer-_BioNTech_Vaccine_Analysis_Print.pdf

Improving the adjuvanting activity of mRNA vaccines

As stated earlier, mRNA vaccines have inherent adjuvant properties that can be beneficial or harmful.
Therefore, in the field of infectious diseases, specific adjuvant strategies have been tested to promote immune activation without blocking mRNA expression.
One strategy is to co-formulate mRNA with nucleosides modified with MPLA (monophosphoryl lipid A), a TLR-4 agonist[210] .

As discussed above, the use of modified nucleosides alters the observed type I IFN response, whereas MLPA allows a Better induction of T-cell activation.
A more recent strategy has been to develop double-stranded short regions in the poly-A tail[211] or in the 3'UTR [212] of mRNA.
Hybridization of a short poly-U RNA into mRNA does not substantially reduce the transcript translation efficiency in the DC.
Compared with mRNA alone, the poly-A/poly-U transcript induces 10-100-fold IFN-β and IL-6, and upregulates co-stimulatory molecules involved in DC activation and migration (CD40, CD86) and the chemokine receptor (CCR7); in addition, the poly-A/poly-U dsRNA region is recognized by TLR-3 and RIG-I, but not by MDA-5.

The use of adjuvant in mRNA vaccines is increasing, but it should be used with caution as it may be counterproductive, especially when immunostimulatory molecules are used because of their close interaction with the innate immunity pathway.

Impact of type I IFN in mRNA vaccination

Activation of PRRs by mRNA after vaccination generally leads to strong production of type I IFN, the main agent modulating mRNA vaccine efficiency.
The scientific community is still debating whether an increase in the level of type I IFN is beneficial or harmful in such context because of its "Janus" effect.
In fact, it can be positive for its stimulatory properties, leading to a productive immune response, or negative when it blocks mRNA translation and dampens the immune response.

For example, DCs incubated with type I IFN show over-regulation of co-stimulatory molecules that promote antigen presentation[213] , while *in vivo* and depending on the timing of type I IFN signaling, the

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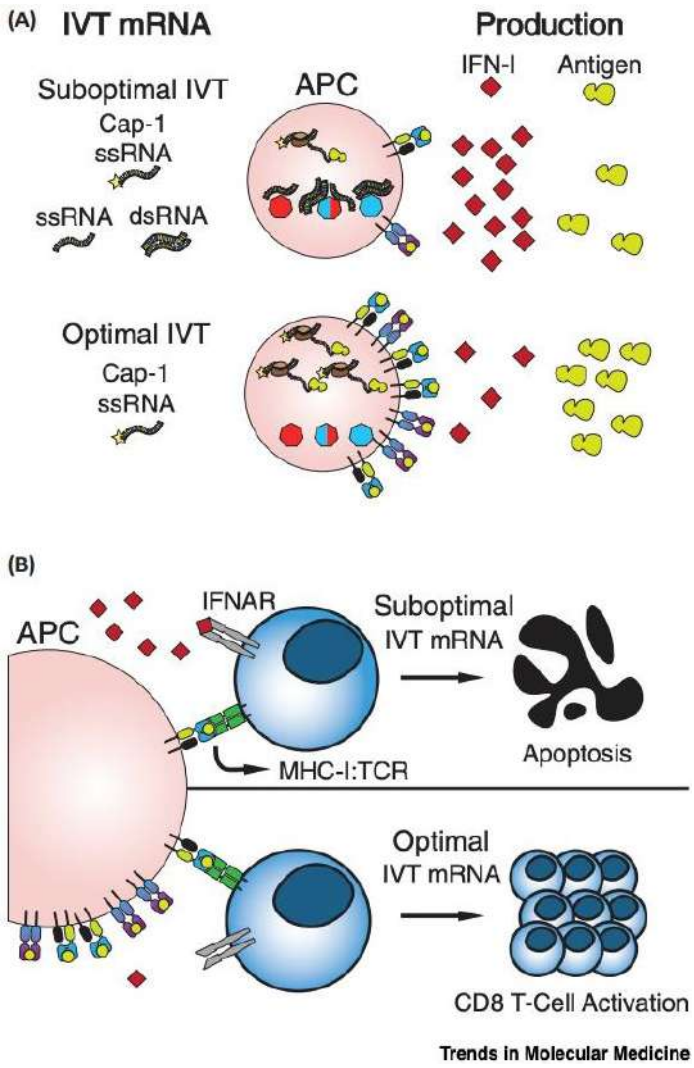
PLoS Biol. 2014 Jan;12(1):e1001759. doi: 10.1371/journal.pbio.1001759. Epub 2014 Jan 7. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3883643/

CD8 T cells can be activated (increasing cell activation and proliferation) or inhibited (leading to apoptosis).[214]



Interaction of IVT-mRNA vaccines and type I interferon (IFN) with regard to antigen production and T-cell responses.
(A) In the presence of double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) (suboptimal IVT-mRNA populations), pattern recognition receptor (PRR) overactivation leads to inhibition of the translation mechanism and overproduction and secretion of IFN-I (red square) into the extracellular environment. Optimal and capped IVT-mRNAs do not activate PRRs, leading to low IFN-I production and high antigen expression (yellow) and presentation in APCs.
(B) When suboptimal IVT-mRNAs are used (upper panel), naive CD8 T cells (blue) are primed first by IFN-I and receive a second T-cell receptor (TCR)-MHC-I signal, leading to T-cell apoptosis [89,90]. When optimized IVT-mRNAs are used (lower panel), an opposite effect could be hypothesized, naïve CD8 T cells are triggered first by TCR- MHC-I signal and then by IFN-I, leading to CD8 T cell activation and proliferation.
Abbreviation: IFNAR, type I IFN receptor.

https://www.cell.com/trends/molecular-medicine/fulltext/S1471-4914(19)30244-8

Based on its autoadjuvant effect, vaccine mRNA may exhibit some properties similar to viral mRNA in that it can be recognized by antigen-presenting cells (APCs), which subsequently activate pattern recognition receptors (PRRs) such as Toll-like receptors 3 (TLR3), TLR7 and TLR8. [215]

[214] De Beuckelaer A, Grooten J, De Koker S.
Type I Interferons Modulate CD8+ T Cell Immunity to mRNA Vaccines.
Trends Mol Med. 2017 Mar;23(3):216-226. doi: 10.1016/j.molmed.2017.01.006. Epub 2017 Feb 7.
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Double-stranded RNA (dsRNA) can combine with some retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in the cytoplasm, such as RIG-I and 5 associated with melanoma differentiation (MDA5), which promotes APC maturation, pro-inflammatory cytokine secretion, and type I interferon (IFN) secretion. [216] This leads to strong humoral and cellular antigen-specific immune responses.
Single-stranded RNA (ssRNA) can trigger the antiviral activation state of dendritic cells through recognition of TLR7 and TLR8 during *in vivo* mRNA transmission. [217]
Contaminants of dsRNA can also trigger immune activation through recognition of TLR3s. [218] However, an excessive immune response stimulated by mRNA in the cytoplasm can cause cells to secrete large amounts of type I IFN and other interferons that can inhibit mRNA translation and eventually lead to translational stagnation, RNA degradation, reduced activation of CD8 T lymphocytes^{+} and finally blockage of the immune response. [219] As already seen, this could adversely affect some applications of mRNA such as vaccines and protein replacement therapies.

[216] De Beuckelaer A, Grooten J, De Koker S.
Type I Interferons Modulate CD8+ T Cell Immunity to mRNA Vaccines.
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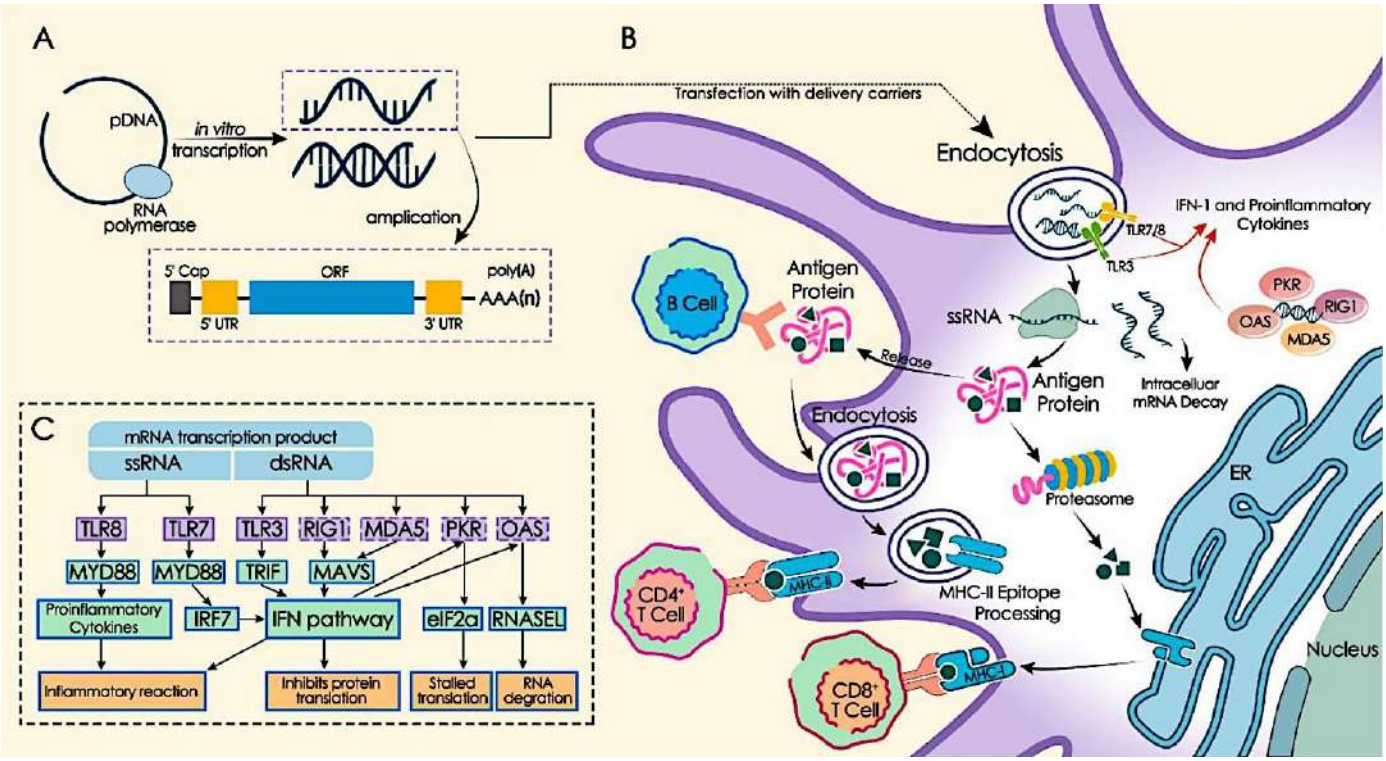
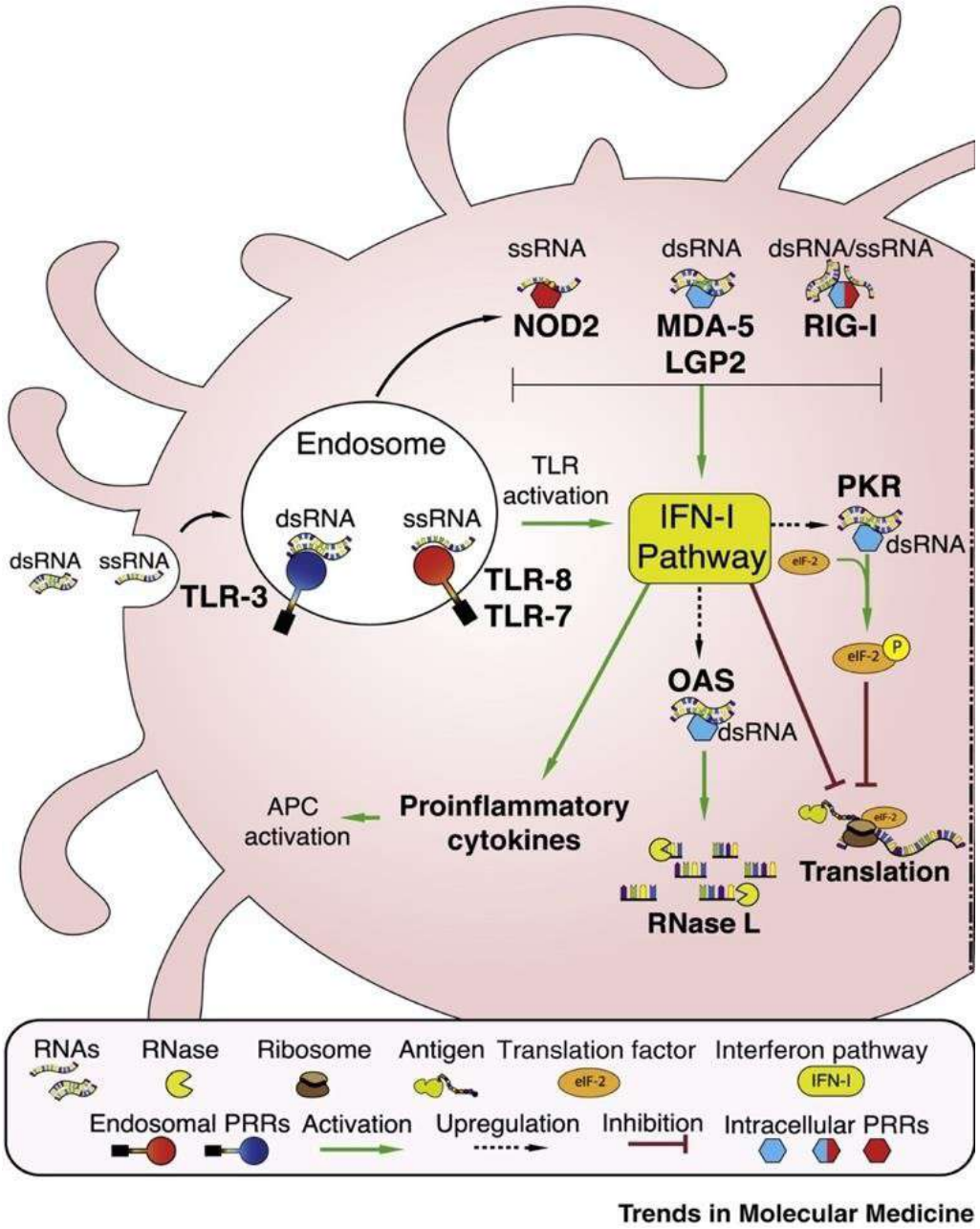


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7554980/

In vitro mRNA transcription and activation of innate immunity. (A) In vitro mRNA transcription. Using DNA with antigen coding sequence as a template, in vitro mRNA transcription products contain single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), etc. The ssRNA structure normally includes five-prime cap (5 ' cap), five-prime untranslated region (5 ' UTR), open reading frame (ORF) region, three-prime untranslated region (3 ' UTR) and poly (A) tail structure. (B) RNA translation and antigen presentation. Through endocytosis, mRNAs enter the cytoplasm. Some mRNAs combine with host cell ribosomes and are successfully translated. Antigenic proteins can be degraded into antigenic peptides by the proteasome in the cytoplasm and presented to cytotoxic T lymphocytes (CTLs) through the major histocompatibility complex (MHC) I pathway. Or they can be released from the host cell and taken up by DCs. Then, they are degraded and presented to helper T lymphocytes and B lymphocytes via the MHC-II pathway. B cells can also recognize the proteins of the released antigen. (C) Autoadjuvant effect. Various pattern recognition receptors (PRRs) can recognize the mRNA transcription product in vitro. ssRNA can be recognized by endosomal innate immune receptors (e.g., Toll-like receptor 7 (TLR7), TLR8). dsRNA can be recognized by endosomal innate immune receptors (e.g., Toll-like receptor 7 (TLR7), TLR8), TLR3) and by cytoplasmic innate immune receptors (e.g., activated protein kinase RNA (PKR), retinoic acid-induced gene protein I [21] cible (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and 2'-5'-oligoadenylate synthase (OAS). Based on these, mRNA products can stimulate the secretion of proinflammatory cytokines and type I interferon (IFN), which leads to antigen presentation cell activation (APC) and inflammatory reaction, but they can also activate antiviral enzymes that cause stalling of mRNA translation and mRNA degradation.



https://www.cell.com/trends/molecular-medicine/fulltext/S1471-4914(19)30244-8

RNA Sensing and Innate Immunity Activation.

After cellular uptake, RNA can be recognized by different sets of pattern recognition receptors (PRRs); endosomal (bulb-like structure) or cytosolic (hexagons) specific for single-stranded RNA (ssRNA, red) and double-stranded RNA (dsRNA, blue). Upon RNA detection, PRRs can lead to activation of the interferon type I (IFN-I) pathway characterized by upregulation of hundreds of genes, including those encoding proinflammatory cytokines, RNA-dependent protein kinase (PKR) and oligoadenylate receptor synthetase (OAS), but also inhibition of translation and mRNA degradation. Production of proinflammatory cytokines and chemokines leads to activation of antigen-presenting cells (APCs). Abbreviations: MDA-5, 5 associated with melanoma differentiation; NOD2, nucleotide oligomerization domain 2; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor.

TOXICOLOGY OF mRNA VACCINES.

In the following section, the mechanisms of damage induction of the two mRNA vaccine components: liposomes and mRNA will be discussed in detail.

Risk assessment

Cancer mRNA immunotherapies have taken a primary role in the use of mRNA-based therapies, and this has allowed for faster progress in the field of prophylactic vaccines.

However, researchers should be cautious when data are extrapolated from cancer to prophylactic vaccines for infectious diseases.
Vaccine safety assessment (risk-benefit) in cancer vaccines is more permissive than in prophylactic vaccines, furthermore, a strong IFN-I response may aid cancer clearance, and the route of administration may be very different (intravenous, intranodal or intratumoral in cancer vaccines vs intramuscular, intradermal or subcutaneous in prophylactic vaccines).

Because of the role of innate immunity in the quality of vaccine immune responses, each delivery platform and mRNA design must perfectly match the targeted pathogen to ensure efficacy.
A vaccine that requires a strong T-cell response (intracellular pathogens) will not lead to the same immune response with long-lasting neutralizing antibodies (extracellular pathogens).

Although the mRNA vaccine platform has provided safety data considered adequate by regulatory agencies, adverse effects may still occur: the spread of mRNA molecules into the extracellular space, the generation of anti-mRNA antibodies, or the induction of chronic autoimmune diseases could jeopardize the field of mRNA vaccines if these risks are not carefully addressed.

Some strategies to minimize the risk of generating anti-mRNA antibodies could be decreasing the amounts of mRNA, limiting the presence of mRNA in the extracellular space, or using fewer immunogenic modified nucleosides.
Before entering clinical trials, in vitro and in vivo data must be screened for reactogenicity and tolerogenicity to ensure that innate immune responses and vaccine design have been adequately addressed.

Because these responses are highly sensitive, it is important to evaluate each delivery system, formulation or route of administration in relevant animal models on a case-by-case basis to prevent potential adverse effects.
Several clinical trials of mRNA vaccines have shown adverse effects including myopathy, lactic acidosis, pancreatitis, lipodystrophy, hepatic steatosis, and nerve tissue damage. Therefore, in addition to mRNA per se, some vehicle elements should be screened for toxicity, e.g., LNPs showed liver toxicity in a clinical trial using therapeutic mRNA for Crigler-Najjar syndrome.
The induction of innate immunity by mRNA sequences is the main barrier to the design of efficient and safe mRNA vaccines. [220]

Safety of mRNA vaccines against infectious diseases

Because the mRNA production process does not require toxic chemicals or cell cultures that could be contaminated with adventitious viruses, mRNA production avoids common risks associated with other vaccine platforms, including live viruses, viral vectors, inactivated viruses, and subunit protein vaccines.
In addition, the short mRNA production time presents little opportunity to introduce contaminating microorganisms.
In vaccinated people, the theoretical risks of infection or integration of the vector into the host cell DNA are not considered a problem for mRNA, and therefore mRNA vaccines have been considered a relatively safe vaccine format.

However, recent human studies have shown moderate and in some cases severe systemic or injection site reactions for several mRNA platforms. [221]

[220] https://pubmed.ncbi.nlm.nih.gov/31699497/

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5475249/

Potential safety issues that could be evaluated in future preclinical and clinical studies include local and systemic inflammation, biodistribution and persistence of the expressed immunogen, stimulation of autoreactive antibodies, and potential toxic effects of any nonnative nucleotides and components of the delivery system.

One possible concern could be that some mRNA-based vaccine platforms induce potent type I interferon responses associated not only with inflammation but also with autoimmunity[222] .
By stimulating dendritic cell maturation and eliciting robust T and B cell responses, mRNA vaccines can activate autoreactive lymphocytes and reactivate autoimmune diseases.
Therefore, identifying individuals at increased risk of autoimmune reactions before mRNA vaccination should include taking appropriate precautions. [223]

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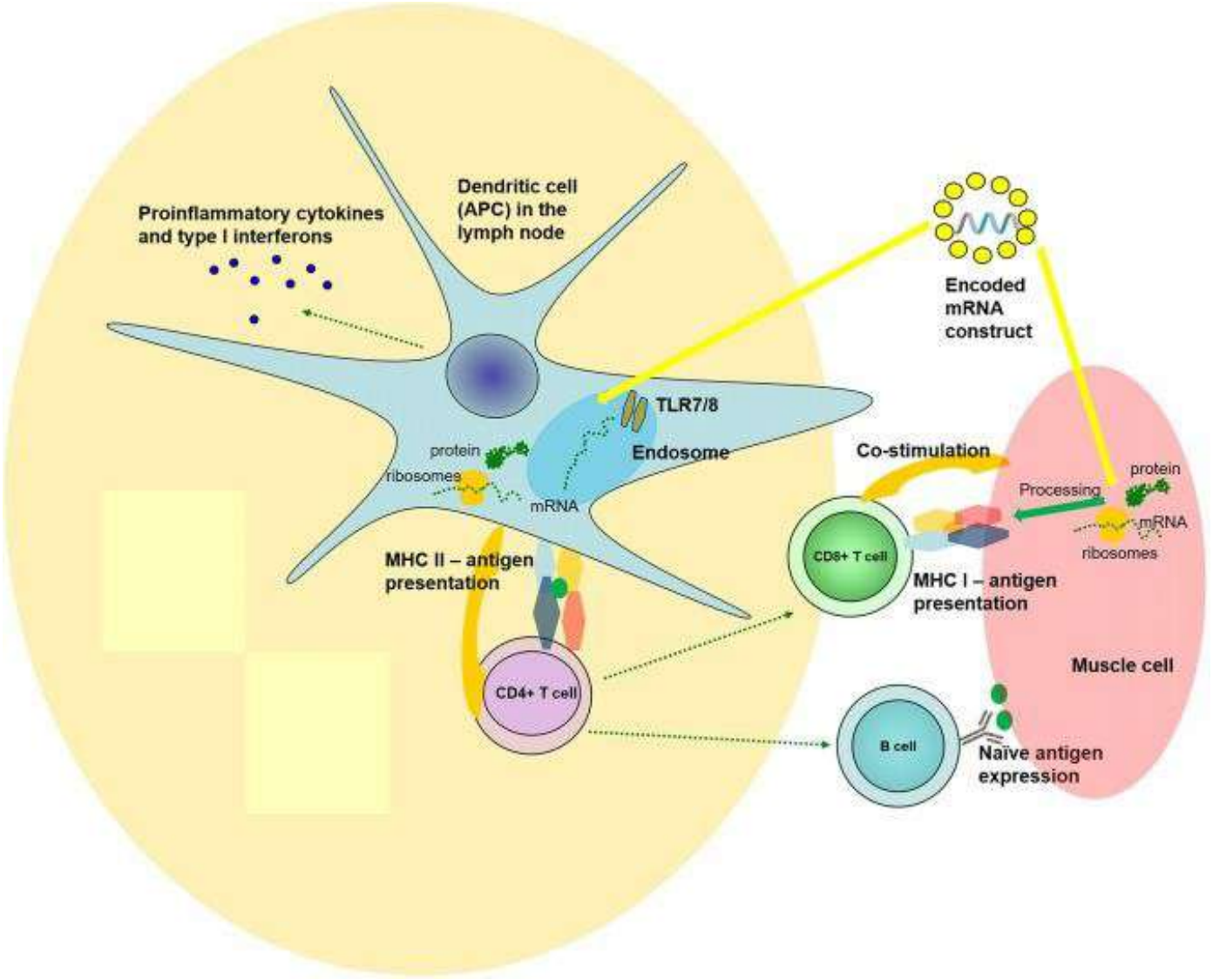

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7846902/
The immune processes involved in the mechanism of mRNA vaccines: activation of helper T cells ($CD4^+$) by MHC I molecules and processed viral antigen in antigen-presenting cells in the lymph node; stimulation of cytotoxic T cells ($CD8^+$) by MHC class II molecules and processed viral antigen and B cells by native viral antigens. In antigen-presenting cells, mRNA detects TLR7 and 8, leading to activation of the downward cascade and production and secretion of proinflammatory cytokines and type I interferons. Some of the mechanisms are simplified in the figure by omission (i.e., the inflammasome, proteasome, secondary messengers, etc.).

Another potential safety issue, as mentioned above, could arise from the presence of extracellular mRNA during vaccination. Naked extracellular RNA has been shown to increase the permeability of tightly packed endothelial cells and thus may contribute to edema.[224]

Other studies have shown that extracellular RNA, acting as a DAMPS, also promotes pathological thrombus formation, and cardiomyocyte death[225] .

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Extracellular RNA mediates endothelial-cell permeability via vascular endothelial growth factor.
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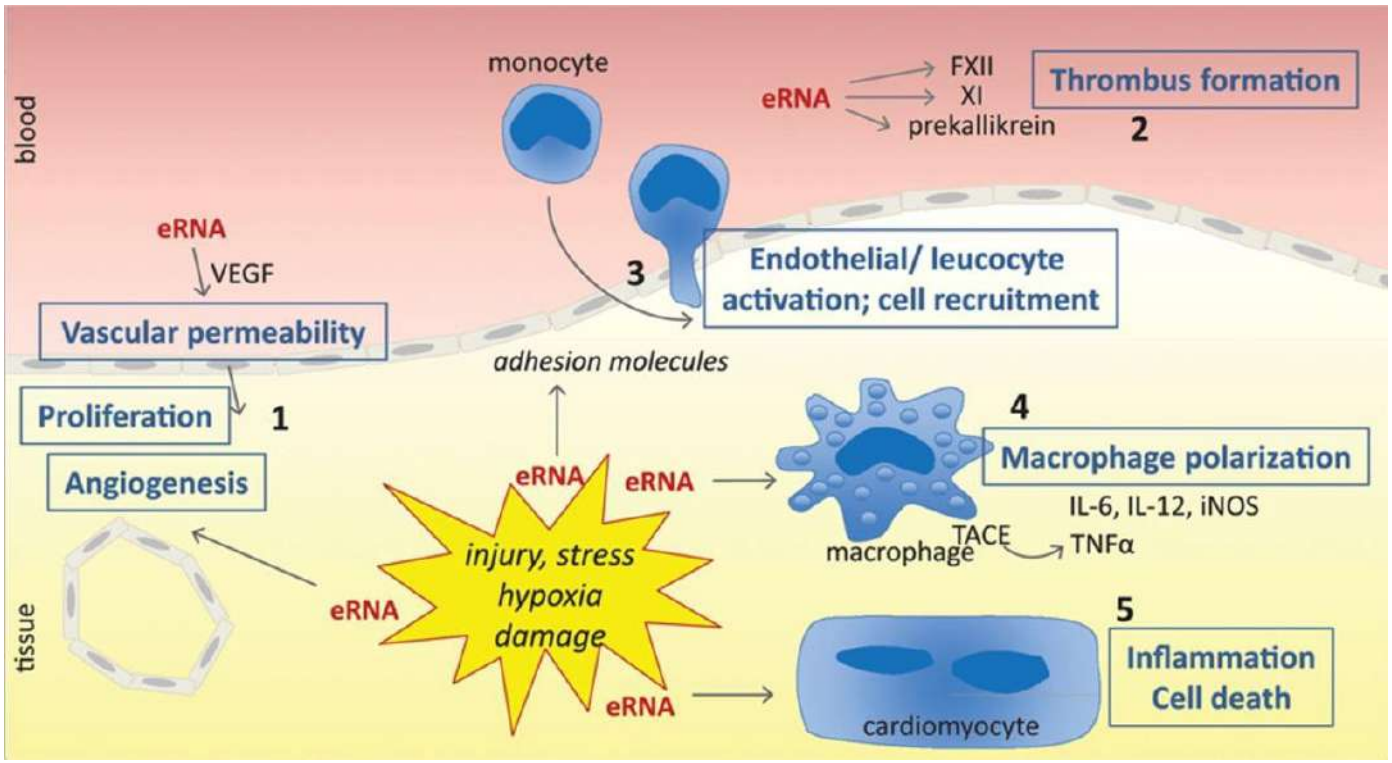

https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.115.307961
Contribution of extracellular RNAs (eRNAs) to vascular homeostasis and cardiovascular disease. After cellular stress (such as hypoxia, injury, and exposure to pathogens), eRNA is found to be expressed at various extracellular sites, both intravascular and extravascular. In addition, eRNA can be derived from damaged or apoptotic cells or is released upon activation of inflammatory cells and accumulates within atherosclerotic plaques in the course of disease development. The following functional activities of eRNA are promoted by direct interactions with specific extracellular proteins: (1) Promotion of vascular hyperpermeability by eRNA as a coreceptor/cofactor of vascular endothelial growth factor (VEGF) and by the induction of vasculogenesis/angiogenesis. (2) Initiation of intrinsic blood coagulation (via contact phase proteins) and thrombus formation. (3) Induction of leukocyte recruitment to the inflamed vessel wall by eRNA-mediated elevation of intercellular adhesion molecule 1 expression in endothelial cells or vascular cell adhesion molecule 1 and P-selectin in vascular smooth muscle cells. (4) Orientation of monocytes/macrophages toward the M1 proinflammatory phenotype, accompanied by upregulated expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12, inducible nitric oxide synthase (iNOS) and response factors, such as chemokines. Here, eRNA triggers TNF-α converting enzyme (TACE) to release functionally active TNF- α. (5) Promotion of cardiomyocyte death by inducing a hyperinflammatory cascade involving TNF-α in the context of ischemia-reperfusion injury.

Safety will therefore require ongoing evaluation as different mRNA modalities and delivery systems are used for the first time in humans and are tested on larger patient populations.

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Foley JH, Conway EM.
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Insight **The mechanisms of anaphylaxis [226]**

In 2003, to harmonize allergy nomenclature worldwide, the World Allergy Organization (WAO) proposed two classifications of anaphylaxis based on the pathophysiological mechanism involved in the reaction.

The term **allergic anaphylaxis** denotes reactions mediated by an immunologic mechanism, related to IgE, immunoglobulins, or the immune complement complex (corresponding to the classic hypersensitivity reactions [HSR] described by Gell and Coombs).

The term **nonallergic anaphylaxis** denotes reactions mediated by other mechanisms (e.g., direct activation by bradykinin or complement), which are usually triggered by agents or events that induce sudden mast cell or basophil activation.[227]

Phenotypes are defined by clinical presentation, and *endotypes* refer to the cellular and molecular mechanisms of HSRs defined by diagnostic biomarkers (skin test, tryptase, IgE, interleukin-6 and others).

The phenotypes of anaphylaxis are classified, based on their clinical presentation, into type I reactions, cytokine release reactions (CRRs), mixed reactions, and, finally, bradykinin and complement-like reactions. The corresponding endotypes underlying these phenotypes include IgE- and non-IgE-mediated mechanisms, cytokine-mediated mechanisms, mixed processes, and direct activation of immune cells by complement or bradykinin.[228]

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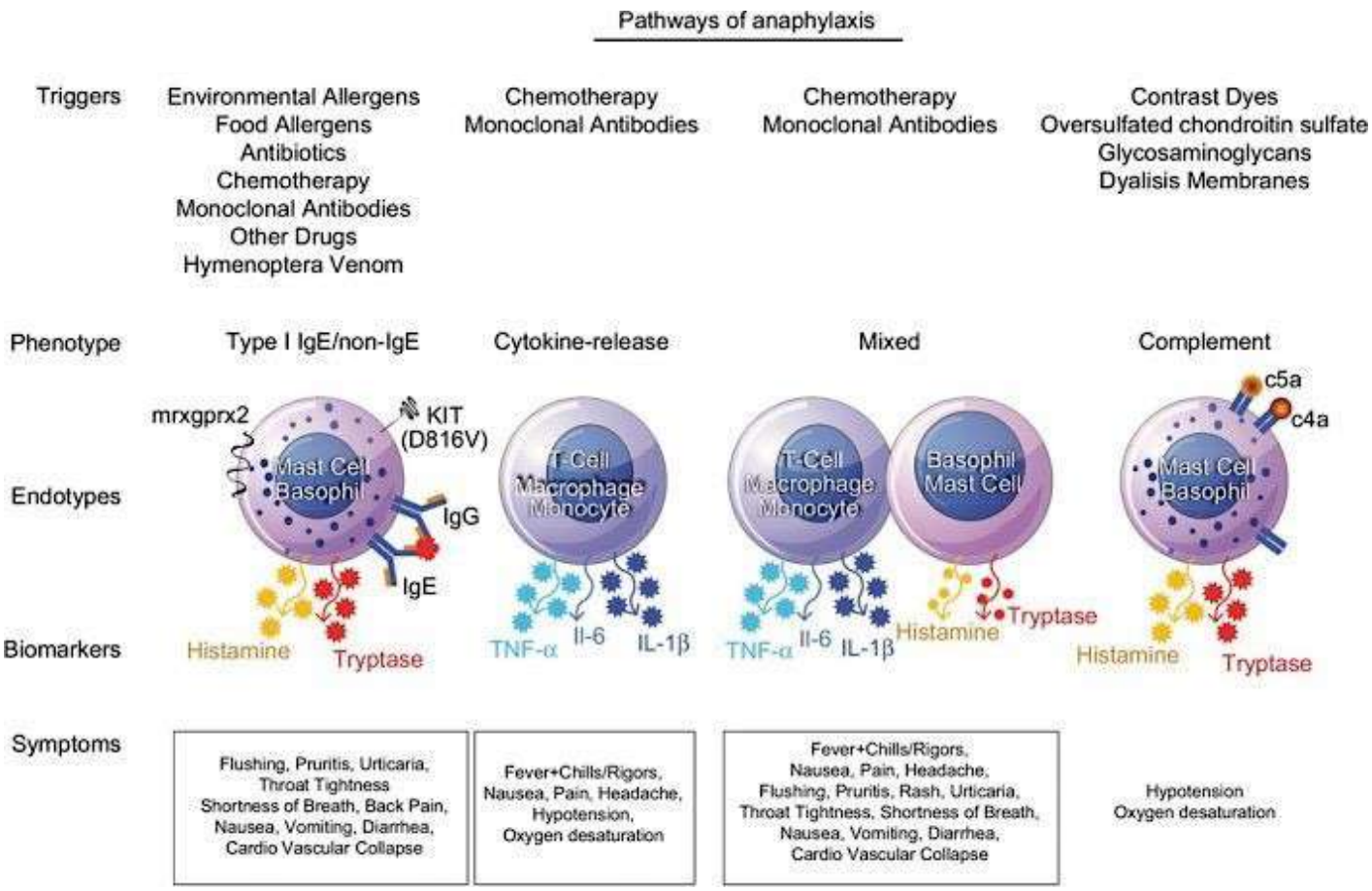

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7361272/

Type I reactions.

IgE-mediated anaphylaxis is the main mechanism underlying allergic anaphylaxis. [229]

After exposure to the allergen, a series of signals triggers the production of allergen-specific IgE by B cells (sensitization phenomenon).

In subsequent exposures, the antigen-allergen-specific IgE complex binds to the Fcε-RI receptor on mast cells and/or basophils and, with appropriate signaling, activates and degranulates these cells, thereby releasing preformed mediators, enzymes, and cytokines and facilitating the synthesis of *de novo* inflammatory mediators (e.g., tryptase, histamine, leukotrienes, prostaglandins, platelet-activating factor [PAF], cytokines) .[230]

Mediators cause allergic symptoms by acting directly on tissues. The reaction spreads by recruiting and activating additional inflammatory cells, particularly eosinophils, which release more mediators, including lipid-derived mediators such as prostaglandin D2 and cysteinyl leukotrienes. [231]

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In addition to the classic IgE-mediated pathway, other possible pathways have been described in animal models. [232]
One of these alternative pathways is similar to the IgE-mediated pathway but involves IgG antibodies.
IgG-mediated reactions are mediated by IgG complexes that cross-bind to the low-affinity macrophage receptor (Fcγ-RIII) thereby stimulating PAF release (rather than histamine).[233]

PAF causes platelet aggregation and the release of the potent vasoconstrictor thromboxane A2 and serotonin; acts directly on vascular endothelial cells to increase vascular permeability; decreases cardiac output, resulting in hypotension and cardiac dysfunction; and increases smooth muscle contraction of the airways, intestines, and uterus. [234]

Although IgG-dependent anaphylaxis has not been demonstrated in humans, it has been hypothesized that IgG antibodies may mediate systemic anaphylaxis if large numbers of IgG and antigen are present. [235]
IgG receptors are able to activate macrophages[236] and neutrophils[237] to secrete PAF and activate mast cells[238] to induce anaphylaxis in humans. [239]

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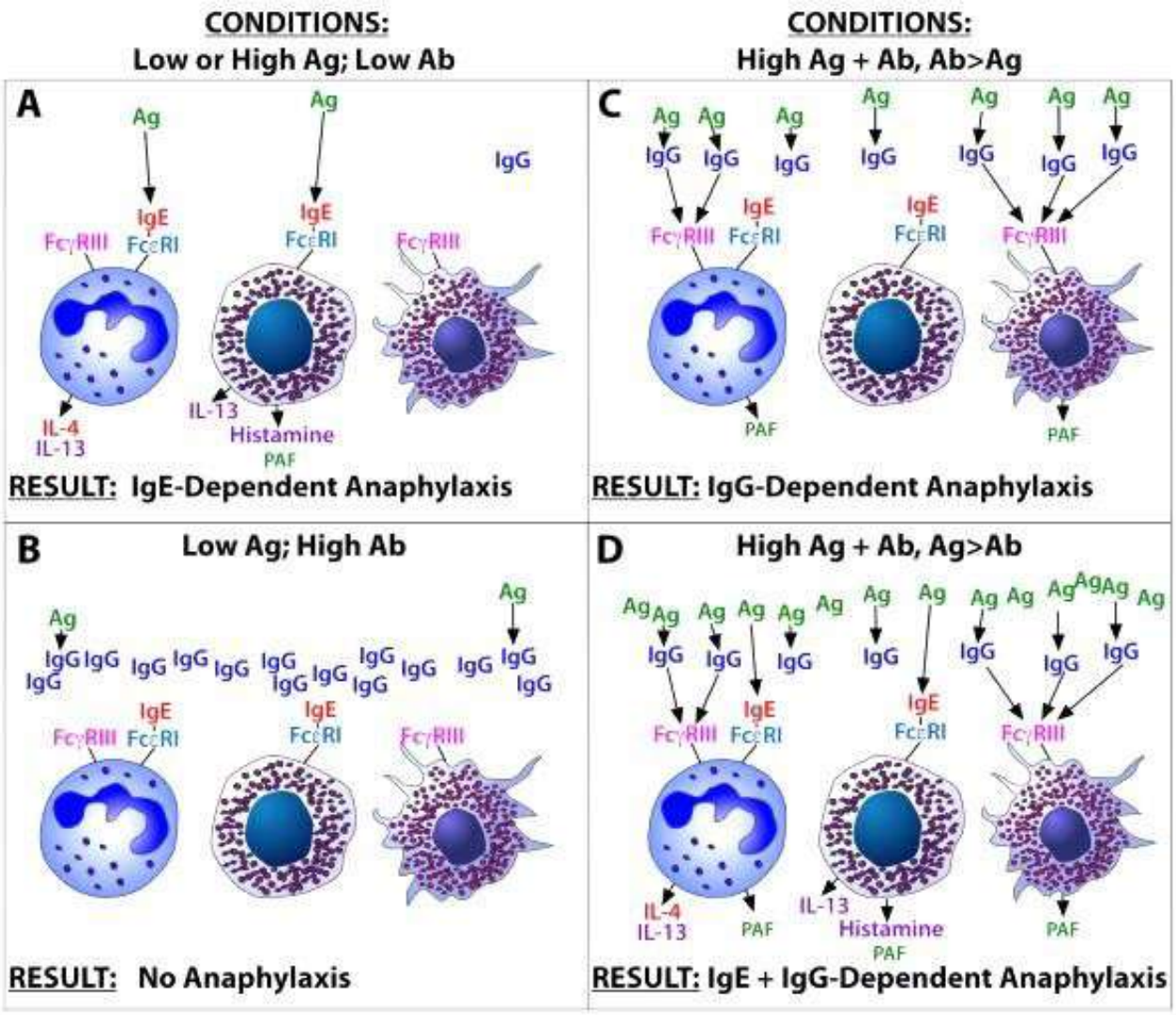
Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions.

Chimeric IgG monoclonal antibodies (mAbs), such as rituximab, have been shown to induce anaphylaxis even in the absence of IgE, suggesting IgG-dependent anaphylaxis. [240]



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The relative concentrations of Ag and Ab determine the role of IgE and IgG Abs in Ab-mediated anaphylaxis
IgE-mediated anaphylaxis requires much less Ab and Ag than IgG-mediated anaphylaxis. Consequently, when Ab levels are low (A), only IgE-mediated anaphylaxis can occur. When Ag levels are low but Ab levels are high (B), IgG "blocking" Abs prevent IgE-mediated anaphylaxis by intercepting Ag before it can bind to FcεRI-associated IgE and bind to the inhibitory receptor, FcγRIIB, but the amount of I IgG/Ag complexes are too low to trigger IgG-mediated anaphylaxis. As a result, anaphylaxis does not occur. When Ag and Ab levels are both high, but Ab is in excess of Ag (C), IgG Abs block Ag binding to FcεRI-bound IgE but IgG/Ag complexes can bind to FcγRs; consequently, only IgG-mediated anaphylaxis occurs. When Ag and Ab levels are both high but Ag is in excess (D), IgG/Ab complexes are sufficient to trigger IgG-mediated anaphylaxis and enough Ag escapes IgG blockade to bind to FcεRI-associated IgE and trigger IgE-mediated anaphylaxis.

Recent reports regarding direct mast cell activation, independent of IgE-mediated activation, indicate that the human G protein-coupled receptor - MRGPRX2 - may be the receptor for many drugs and cationic proteins, such as quinolone antibiotics (p. ciprofloxacin, levofloxacin), general anesthetics such as atracuronium and rocuronium, icatibant, and other drugs with the tetrahydroisoquinoline group (THIQ). [241]

The endotype of IgE-mediated reactions is the release of mediators from mast cells and basophils causing flushing, itching, urticaria, angioedema, shortness of breath, wheezing, nausea, vomiting, diarrhea, hypotension, oxygen desaturation, and cardiovascular collapse along with other symptoms. [242]
Common triggers of these reactions include foods, drugs, latex, hymenoptera venoms and environmental allergens. [243]

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There are important geographic and age-related variations among countries; however, the most common food allergens are peanuts, milk, eggs, nuts, shellfish, fruits, and vegetables. [244]
Antibiotics such as β-lactams, nonsteroidal anti-inflammatory drugs (NSAIDs), chemotherapeutic agents such as platinum derivatives and taxanes, chimeric humanized and human monoclonal antibodies (mAb), general anesthetics, and immunotherapy allergens are other common allergens in both children and adults.[245]

Cytokine release reactions (CRRs)

The CRR phenotype is caused by the release of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL- 1B and IL-6, and their target cells (endotype) include monocytes, macrophages, mast cells and other cells with the Fc gamma receptor (FcγR), an essential participant in many effector functions of the immune system, including the release of inflammatory mediators and antibody-dependent cellular cytotoxicity.

Triggers for these reactions include chimeric, humanized, and human mAbs and chemotherapeutic agents, including oxaliplatin. These drugs have not only been used to treat neoplastic, autoimmune, and inflammatory diseases, but also to treat allergic disorders including allergic asthma, eosinophilic asthma, and chronic urticaria.[246] HSRs to biological agents are less common than standard infusion reactions and vary according to the biological agents involved. [247]
Phenotypic symptoms include chills, fever, and pain that respond to ibuprofen and fluids and have been clinically correlated with IL-6. [248] CRRs are typically not as severe as cytokine storm reactions.

Cytokine storm reactions are acute, severe, and life-threatening systemic complications due to the production of large amounts of cytokines and chemokines, which play a pathological role in the development of symptoms

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systemic[249] , IL-6 and other inflammatory cytokines such as IL -8, TNF-α, IFN-γ and IL-1β induce the inactivation of cadherin, which mediates cell adhesion, leading to vascular leakage by increased capillary permeability; it also induces the formation of tissue factor (thromboplastin) on the cell surface of monocytes, resulting in activation of the extrinsic coagulation pathway. [250]
The effects of inflammatory cytokines play a pathological role in the development of pain, tissue hypoxia, hypotension , myocardial dysfunction, disseminated intravascular coagulation (DIC), and multiorgan dysfunction.
IL-6 is an excellent biomarker of cytokine storm reactions because of its correlation with reaction severity and its longevity in blood serum. [251]

This phenotype is characterized by chills, fever, and generalized malaise followed by hypotension, desaturation, and cardiovascular collapse. [252]
Premedication with anti-inflammatory COX-1 inhibitors and corticosteroids can reduce the intensity of these symptoms but does not protect against severe reactions. [253]

Mixed Reactions (Type I / CRR)

Mixed reactions occur as a mixture of type I and CRR phenotypes and, typically, are observed during chemotherapy and/or HSR with mAbs, in which symptoms of IgE-mediated reactions such as flushing, itching, urticaria, angioedema, difficulty breathing, wheezing, nausea vomiting, diarrhea, hypotension, desaturation, cardiovascular collapse, and life-threatening anaphylaxis, which occur secondary to the release of mast cell/basophilic mediators (tryptase, histamine, leukotrienes, and prostaglandins), overlap with symptoms secondary to cytokine release

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https://pubmed.ncbi.nlm.nih.gov/27381687/

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Diagnosis and management of anaphylaxis in precision medicine.
J Allergy Clin Immunol. 2017 Aug;140(2):321-333. doi: 10.1016/j.jaci.2017.06.012.
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Immunol Allergy Clin North Am. 2015 May;35(2):335-48. doi: 10.1016/j.iac.2015.01.011.
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[253] Castells M.
Diagnosis and management of anaphylaxis in precision medicine.
J Allergy Clin Immunol. 2017 Aug;140(2):321-333. doi: 10.1016/j.jaci.2017.06.012.
https://pubmed.ncbi.nlm.nih.gov/28780940/

proinflammatory and chemokines (IL-1β, IL-6, and TNF-α) such as chills, fever, malaise, hypotension, desaturation, and cardiovascular collapse, thus making it impossible to distinguish the mechanisms.

Complementary/bradykinin-like reactions

Complement reactions involve direct activation of mast cells and other immune cells through complement activation as well as direct and indirect activation of the intrinsic coagulation pathway.[254]
Immune complexes can activate the complement system, generating anaphylatoxins such as C3a and C5a, which can bind to complement receptors resulting in the release of histamine, leukotrienes, and prostaglandins that can induce flushing, urticaria, hypoxia, vasodilation, and hypotension. [255]
This mechanism has been described with drugs such as vancomycin,[256] contrast media,[257] dialysis membranes , and infusions of drugs that are suspended in certain lipid vehicles such as Cremophor EL, polysorbate 80, and polyethylene glycol.
[258]

Notably, reports have also suggested that complement may play an important role in vespid-induced anaphylaxis by exacerbating the reaction due to complement activation by proteases in the venom, which is in addition to the IgE-mediated reaction.[259]

The molecular pathway of bradykinin reactions has been elucidated in animal models and involves an increase in heparin and Factor XII-driven contact system that results in bradykinin production and ultimately explains the increased vascular permeability (clinically speaking with hypotension and desaturation). [260]

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[254] Muñoz-Cano R, Picado C, Valero A, Bartra J.
Mechanisms of Anaphylaxis Beyond IgE.
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Sala-Cunill A, et al
Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions.
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https://pubmed.ncbi.nlm.nih.gov/25240785/.

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Expression of the anaphylatoxin receptors C3aR and C5aR is increased in fatal asthma.
J Allergy Clin Immunol. 2005 Jun;115(6):1148-54. doi: 10.1016/j.jaci.2005.01.068.
https://pubmed.ncbi.nlm.nih.gov/15940127/

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Vancomycin anaphylaxis and successful desensitization in a patient with end stage renal disease on hemodialysis by maintaining steady antibiotic levels.
Ann Allergy Asthma Immunol. 2000 Jun;84(6):633-5. doi: 10.1016/S1081-1206(10)62416-7.
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Radiographic contrast media infusions. Measurement of histamine, complement, and fibrin split products and correlation with clinical parameters.
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Weiszhár Z, Czúcz J, Révész C, Rosivall L, Szebeni J, Rozsnyay Z.
Complement activation by polyethoxylated pharmaceutical surfactants: Cremophor-EL, Tween-80 and Tween-20.
Eur J Pharm Sci. 2012 Mar 12;45(4):492-8. doi: 10.1016/j.ejps.2011.09.016. Epub 2011 Sep 22.
https://pubmed.ncbi.nlm.nih.gov/21963457/

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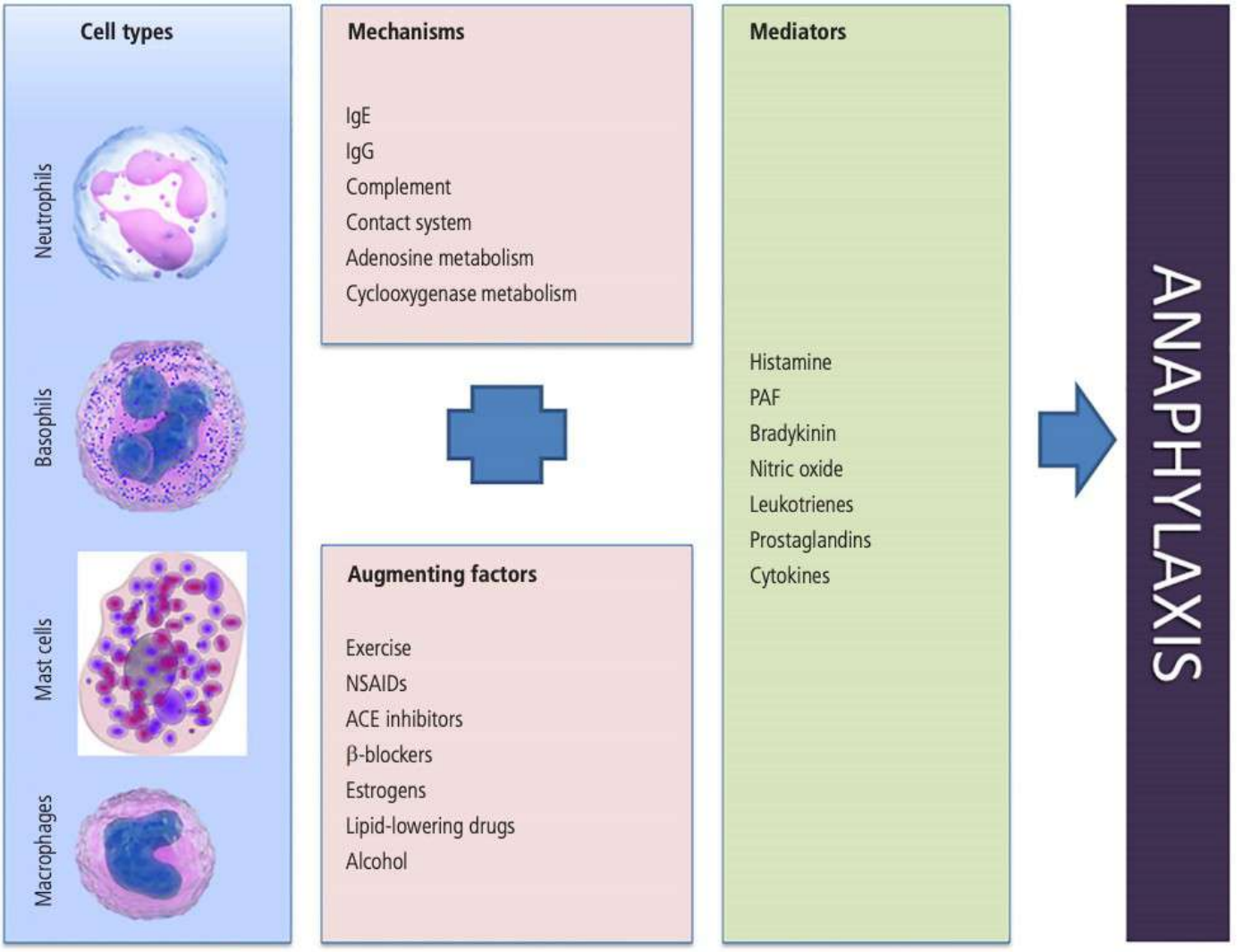
Preliminary report: complement activation in wasp-sting anaphylaxis.
Lancet. 1990 Oct 13;336(8720):904-6. doi: 10.1016/0140-6736(90)92272-j.
https://pubmed.ncbi.nlm.nih.gov/1976931/

[260] Sala-Cunill A, et al
Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions.
J Allergy Clin Immunol. 2015 Apr;135(4):1031-1043.e6. doi: 10.1016/j.jaci.2014.07.057. Epub 2014 Sep 18.
https://pubmed.ncbi.nlm.nih.gov/25240785/

These symptoms have been associated with e.g., contamination of heparin with excessively sulfated chondroitin sulfate.[261]



http://www.jiaci.org/issues/vol26issue2/1.pdf
Mechanism of anaphylaxis. Ig indicates immunoglobulin; NSAID, nonsteroidal anti-inflammatory drug; ACE, angiotensin-converting enzyme; PAF, platelet-activating factor

Toxicology of liposomes

For an in-depth discussion of nanoparticle toxicology, see also Traditional Vaccine Platforms-Part One

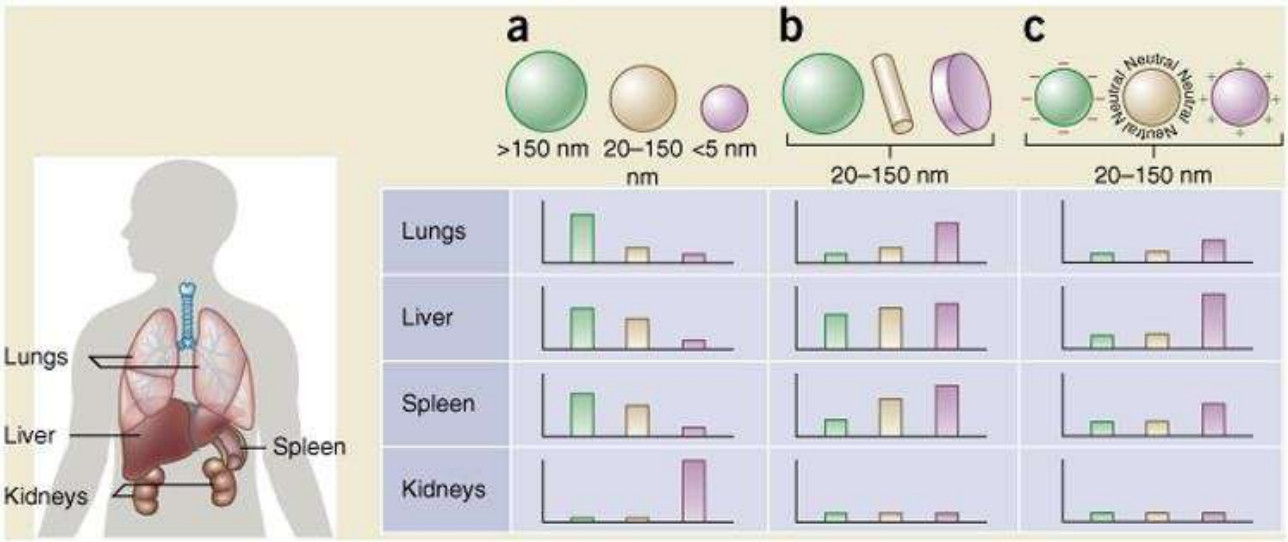
Non-IgE-mediated pseudoallergy (CARPA)

From the toxicological point of view, it should be mentioned that studies on RNAi release through cationic LNPs showed that polyamines such as polyethylenimine and poly-L-lysine led to high serum liver enzyme levels, reduced weight

[261] Kishimoto TK, Viswanathan K, Ganguly T, et al.
Contaminated heparin associated with adverse clinical events and activation of the contact system
[published correction appears in N Engl J Med. 2010 Mar 18;362(11):1056]. N Engl J Med. 2008;358(23):2457-2467. doi:10.1056/NEJMoa0803200
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3778681/

body and dramatically reduced the total leukocyte count, suggesting a mechanism of immunosuppression after intravenous administration.[262]

The following figure shows the biodistribution of nanoparticles according to size, surface charge and shape. Liposomes used in the Pfizer and Moderna vaccine tend mainly to accumulate in the liver, with the increased Risk of hepatotoxicity, (see Assessment report of Comirnaty- preclinical studies of new ingredients)



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4978509/

The size, shape and surface charge of the nanoparticles determine biodistribution among different organs including lungs, liver, spleen and kidneys.

(a) Spherical particles including gold nanoparticles, liposomes and micelles/polymeric nanoparticles can vary in size and show disparate in vivo fates. Large rigid particles with diameters> 2,000 nm easily accumulate within the spleen and liver as well as in the capillaries of the lungs. Nanoparticles in the 100-200 nm range have been shown to extravasate through vascular fenestrations of tumors (EPR effect) and escape filtration by the liver and spleen. As the size increases beyond 150 nm, more and more nanoparticles are trapped in the liver and spleen. Nanoparticles of small size (<5 nm) are filtered out by the kidneys. [263]

(b) New "top-down" and "bottom-up" fabrication techniques have enabled the exploration of different nanoparticle geometries, including cylindrical and discoidal forms, which have been shown to exhibit pronounced effects on pharmacokinetics and biodistribution. Different nanoparticle shapes exhibit unique flow characteristics that substantially alter circulation duration, cell membrane interactions, and macrophage uptake, which in turn affect biodistribution among different organs. [264]

(c) The charge of nanoparticles derived from distinct surface chemicals influences opsonization, circulation times and interaction with resident macrophages of the organs comprising the MPS (mononuclear phagocytic system), with positively charged particles more prone to sequestration by macrophages in the lungs, liver and spleen. Neutral and slightly negatively charged nanoparticles have a longer circulation life and less accumulation in the aforementioned organs of the MPS. [265]

In both b and c, the nanoparticle size is assumed to be between 20 and 150 nm. The individual panels represent the in vivo fate of the nanoparticles, taking into account singular design parameters of size, shape, and surface charge that are independent of each other, which is why the respective scales vary from panel to panel. It is important to note that in vivo biodistribution will undoubtedly vary based on the interaction of many of the above parameters.

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[262] Landesman-Milo D, Peer D.
Toxicity profiling of several common RNAi-based nanomedicines: a comparative study.
Drug Deliv Transl Res. 2014 Feb;4(1):96-103. doi: 10.1007/s13346-013-0158-7.
https://pubmed.ncbi.nlm.nih.gov/25786620/

[263] Longmire M, Choyke PL, Kobayashi H.
Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats.
Nanomedicine (Lond). 2008;3(5):703-717. doi:10.2217/17435889.3.5.703
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3407669/

[264] Black KC, Wang Y, Luehmann HP, et al.
Radioactive 198Au-doped nanostructures with different shapes for in vivo analyses of their biodistribution, tumor uptake, and intratumoral distribution.
ACS Nano. 2014;8(5):4385-4394. doi:10.1021/nn406258m
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4358630/

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The effect of surface charge on in vivo biodistribution of PEG-oligocholic acid based micellar nanoparticles.
Biomaterials. 2011;32(13):3435-3446. doi:10.1016/j.biomaterials.2011.01.021
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3055170/

It is also known that intravenous injection of a variety of nanotechnology-enhanced (liposomal, micellar, polymer-conjugated) and protein-based drugs (antibodies, enzymes) can lead to hypersensitivity reactions (HSRs), also known as infusion or anaphylactoid reactions.
The molecular mechanism of mild to severe allergy symptoms may differ from case to case and is mostly unknown, however, in many cases one of the main causes, or a contributing factor, is activation of the complement (C) system.

The clinical relevance of complement activation-related HSRs, particularly *non-IgE-mediated pseudoallergy (CARPA)*, lies in its unpredictability and occasional lethal outcome. Consequently, there is a medical need to develop laboratory analyses and animal models to quantify CARPA.

It is emphasized that anaphylatoxin-induced mast cell release does not fully explain severe reactions, but a second triggering event may be required on the cells mediating allergies, and it is suggested that CARPA represents a "blood stress" reaction, a systemic struggle of the body against harmful biological and chemical agents through the anaphylatoxin/mast cell/circulatory system axis, analogous to the body's struggle against physical and emotional stress through the hypothalamus/pituitary/adrenal axis.
In both cases the response to a wide variety of harmful effects is oriented in a uniform pattern of physiological changes.[266]

[266] Szebeni J.
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https://pubmed.ncbi.nlm.nih.gov/25124145/

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European Journal of Nanomedicine, vol. 7, no. 3, 2015, pp. 233-244. https://doi.org/10.1515/ejnm-2015-0021
https://www.degruyter.com/document/doi/10.1515/ejnm-2015-0021/html

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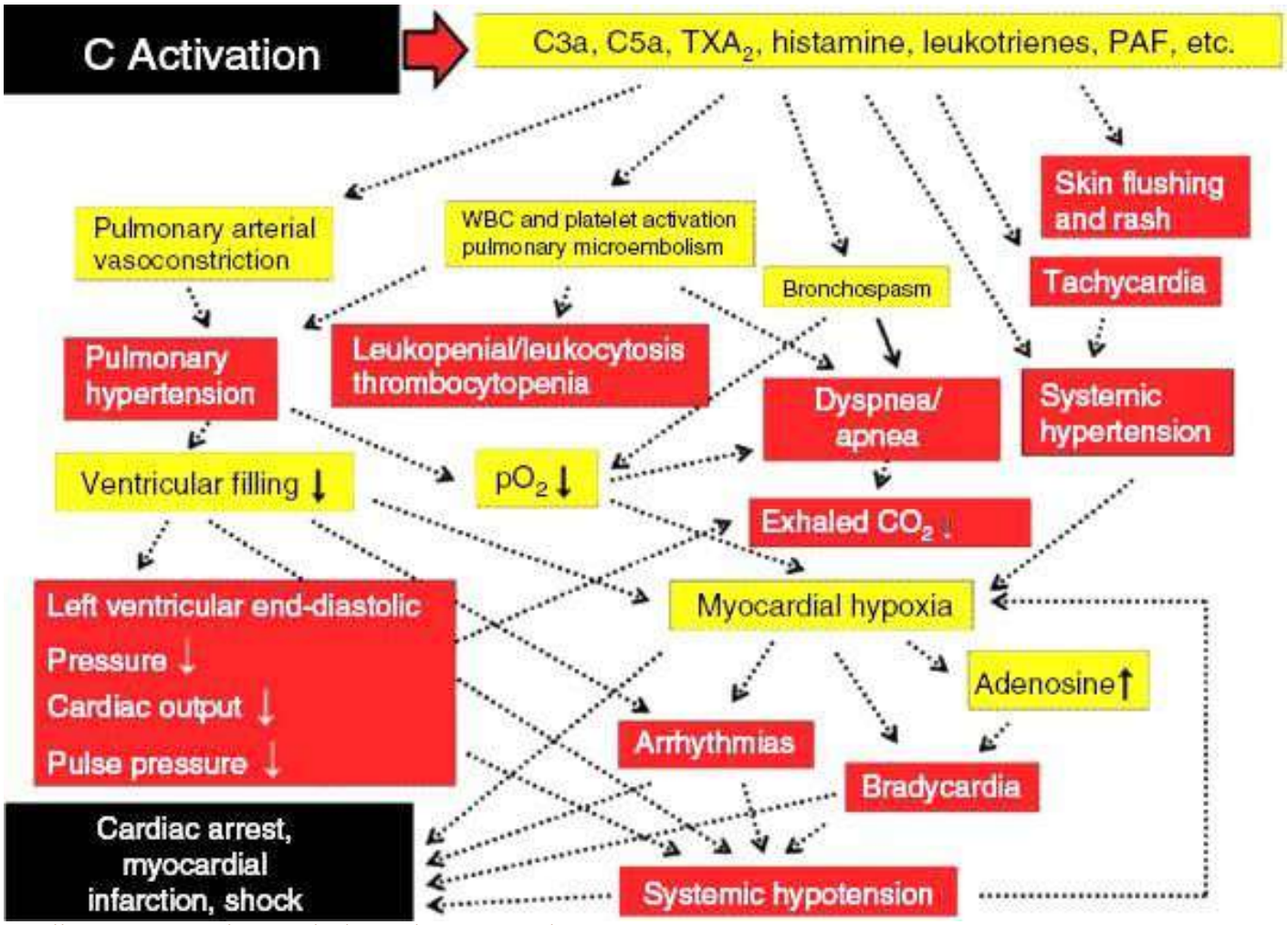
Features of complement activation-related pseudoallergy to liposomes with different surface charge and PEGylation: comparison of the porcine and rat responses. J Control Release. 2014 Dec 10;195:2-10. doi: 10.1016/j.jconrel.2014.08.009.
https://pubmed.ncbi.nlm.nih.gov/25148822/



https://www.degruyter.com/document/doi/10.1515/ejnm-2015-0021/html
Interrelationships among different abnormalities during CARPA, leading to clinical symptoms.

DNA integration

New manufacturing biotechnologies have raised important questions about the safety of new GMO drugs. One of the concerns expressed relates to the potential risk of mRNA integration into human DNA.
It should be noted that while there is a theoretical concern for integration into the host genome regarding plasmid DNA vaccines, this concern is not shared for mRNA-based vaccines for the following reasons:

(1) mRNA remains in the cytoplasm and is not transported to the nucleus.
(2) For integration, the mRNA should be converted into a DNA molecule. Although it is possible for single-stranded DNA to be integrated, the form of DNA integration is generally double-stranded DNA. This requires the presence of a reverse transcriptase and appropriate primers and complementary binding sites on the mRNA to first generate single-stranded DNA and then convert this single-stranded DNA to double-stranded DNA, which still requires appropriate primers and binding sites.
In retroviruses, this process occurs in the retrovirus particle. This is not the case with mRNA vaccines, because although endogenous reverse transcriptase is present in mammalian cells, the enzymes and RNA are not in the appropriate complex to enable efficient reverse transcription.
(3) The final step of retroviral integration requires the activity of viral integrase, which is still in the retroviral particle. It has been shown that integration of the naked DNA double helix is very inefficient.
(4) Vaccine mRNA has been shown to degrade in a relatively short time once absorbed into body cells. Finally, because cellular mRNA is more abundant than mRNA vaccine, it is highly unlikely

that a cellular reverse transcriptase (RT) preferentially copies vaccine mRNA over cellular mRNA. For all these reasons, the risk of integration of mRNA vaccines has been considered negligible. [267]

No, Really, mRNA Vaccines Are Not Going To Affect Your DNA
mRNA Vaccines and COVID-19

It is worth quoting, however, the remarks of Dr. Cimolai N. in his article *"Do RNA vaccines obviate the need for genotoxicity studies?"*[268] on the possibility advanced by manufacturers that RNA vaccines possess the requirements to obviate any concern for cellular transformation and thus may be exempt from the need for regulatory genotoxicity studies [269]

Although the ability of retroviruses to produce retrotranscriptase and integrate into genomic DNA has been known for more than 50 years,[270] the ability of eukaryotic cells to express reverse transcriptase is of more recent acquisition[271] .

[267] Knezevic I, Liu MA, Peden K, Zhou T, Kang HN.
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Vaccines (Basel). 2021 Jan 23;9(2):81. doi: 10.3390/vaccines9020081.
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Human short-term repopulating cells have enhanced telomerase reverse transcriptase expression.
Blood. 2006 Aug 1;108(3):1084-91. doi: 10.1182/blood-2005-09-008904.
https://ashpublications.org/blood/article/108/3/1084/22369/Human-short-term-repopulating-cells-have-enhanced

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Endogenous LINE-1 (Long Interspersed Nuclear Element-1) Reverse Transcriptase Activity in Platelets Controls Translational Events Through RNA-DNA Hybrids.
Arterioscler Thromb Vasc Biol. 2018 Apr;38(4):801-815. doi: 10.1161/ATVBAHA.117.310552. Epub 2018 Jan 4.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5864535/

Holinstat M.
New LINE(s) of Evidence for Genetic Regulation of Platelets.
Arterioscler Thromb Vasc Biol. 2018 Apr;38(4):690-691. doi: 10.1161/ATVBAHA.118.310690.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5951185/

Su Y, Ghodke PP, He M, Li L, Wang Y, Guengerich FP.
Human DNA polymerase η has reverse transcriptase activity in cellular environments.
J Biol Chem. 2019 Apr 12;294(15):6073-6081. doi: 10.1074/jbc.RA119.007925. Epub 2019 Mar 6.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6463694/

The diversity of this class of enzymes as well as their common roles in routine biological processes has been confirmed [272]. In addition, the potential role of the same enzymes in modulating or promoting oncogenesis or cell proliferation has also been the subject of research. [273]
Shimizu et al. further explore this problem with the discovery in cells of cytoplasmic DNA complementary to non-retroviral RNA viruses that may explain the integration of bornavirus and filovirus sequences in the genomes of different mammals. [274]

In addition, there is recently published preprint research in which the integration of SARS-Cov-2 into genomic DNA has been demonstrated, preliminarily and therefore to be confirmed with further, more in-depth studies .[275][276]

The development of stabilized mRNA vaccines can also add another level of complexity. [277] These chemical modifications produce mRNA that is less immunogenic, as unmodified mRNA can be more easily recognized by Toll-like receptors and degraded.[278] Such genetic modifications can make the mRNA more stable or

[272] Spadafora C.
A reverse transcriptase-dependent mechanism plays central roles in fundamental biological processes.
Syst Biol Reprod Med. 2008 Jan-Feb;54(1):11-21. doi: 10.1080/19396360701876815.
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Gladyshev EA, Arkhipova IR.
A widespread class of reverse transcriptase-related cellular genes.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4038843/

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SARS-CoV-2 RNA reverse-transcribed and integrated into the human genome.
bioRxiv [Preprint]. 2020 Dec 13:2020.12.12.422516. doi: 10.1101/2020.12.12.422516.
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Nucleoside Modified mRNA Vaccines for Infectious Diseases.
Methods Mol Biol. 2017;1499:109-121. doi: 10.1007/978-1-4939-6481-9_6.
https://pubmed.ncbi.nlm.nih.gov/27987145/

Jackson NAC, Kester KE, Casimiro D, Gurunathan S, DeRosa F.
The promise of mRNA vaccines: a biotech and industrial perspective.
NPJ Vaccines. 2020 Feb 4;5:11. doi: 10.1038/s41541-020-0159-8.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7000814/

[278] Badieyan ZS, Evans T.
Concise Review: Application of Chemically Modified mRNA in Cell Fate Conversion and Tissue Engineering.
Stem Cells Transl Med. 2019 Aug;8(8):833-843. doi: 10.1002/sctm.18-0259. Epub 2019 Mar 19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6646692/

lead to the production of increased protein expression.[279] Nucleoside modification, however, can also affect secondary RNA structures and may also impact a variety of cellular functions. [280]

Whether such changes in an mRNA vaccine can have a deleterious impact on eukaryotic cell function will have to be tested on a large scale. In the regulatory arena, several research groups have evaluated the safety of vaccines and particularly mRNA vaccines. [281]
While it is generally believed that vaccine formulations do not require genotoxicity studies, newer versions of vaccines such as DNA or RNA vaccines attract a different level of discussion, despite the fact that they are not considered gene therapy products . [282]
More recent specific discussion of COVID-19 vaccines has focused on toxicity on development, reproduction, and biodistribution.[283] The potential for exogenous RNA, viral or otherwise, to integrate into human DNA following vaccination is theoretical to date, as is the potential for any such RNA to induce altered or oncogenic processes in human cells, but until such hypotheses are properly tested following vaccination or natural infection and appropriate studies are published, of which there is a surprising shortage given the importance, regulatory safety assessments for the marketing of RNA vaccines should include genotoxicity studies.

Although nucleic acid and viral vector vaccines do not fall into the category of gene therapy drugs, they are, however, classified as GMO (genetically modified organism) drugs.
The clinical use of both gene therapies (which are also GMOs) and GMO prophylactic vaccines carries the risk that these products may enter the environment through unintentional dispersal or through excretion by the patient, and the GMO could undergo genetic or phenotypic changes, infect, reproduce, remain dormant, compete with existing species, or transfer its genetic material to other species, impacting human health and the environment. Consequently, medicines consisting of or containing a GMO are regulated by environmental and human drug legislation in the European Union (EU), and all its potential risks must be assessed by conducting an environmental risk assessment (ERA) during its development. To conduct a clinical trial with a product based on a GMO, the Sponsor must obtain not only authorization from the ethics committees and relevant national health authorities (NHAs) where the study will take place, but also additional authorization obtained in

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[279] Andries O, Mc Cafferty S, De Smedt SC, Weiss R, Sanders NN, Kitada T.
N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice.
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Parr CJC, Wada S, Kotake K, Kameda S, Matsuura S, Sakashita S, Park S, Sugiyama H, Kuang Y, Saito H.
N 1-Methylpseudouridine substitution enhances the performance of synthetic mRNA switches in cells.
Nucleic Acids Res. 2020 Apr 6;48(6):e35. doi: 10.1093/nar/gkaa070.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102939/

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Regulatory Considerations on the Development of mRNA Vaccines.
Curr Top Microbiol Immunol. 2020 Jul 8. doi: 10.1007/82_2020_220.
https://pubmed.ncbi.nlm.nih.gov/32638114/

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The European Regulatory Environment of RNA-Based Vaccines.
Methods Mol Biol. 2017;1499:203-222. doi: 10.1007/978-1-4939-6481-9_13.
https://pubmed.ncbi.nlm.nih.gov/27987152/

https://www.fda.gov/regulatory-information/search-fda-guidance-documents/development-and-licensure-vaccines-prevent-covid-19

[282] https://www.who.int/biologicals/vaccines/nonclinial_evaluation_of_vaccines/en/

[283] Hager G.
Nonclinical Safety Testing of RNA Vaccines.
Methods Mol Biol. 2017;1499:253-272. doi: 10.1007/978-1-4939-6481-9_16.
https://pubmed.ncbi.nlm.nih.gov/27987155/

following the approval of an ERA by the government authorities in each member state in charge of GMO assessments and responsible for the environment in each country.[284]

Given the public health emergency caused by the COVID-19 outbreak, on July 13, 2020[285] , the European Parliament and the EU Council granted a temporary derogation from these environmental requirements to facilitate clinical trials with GMOs intended to treat or prevent COVID-19, so that they could begin as soon as possible, without the delays generated by the different national implementations of Environmental Directives 2001/18 / EC and 2009/41 / EC and their different requirements[286] .
Although these two Directives aim to ensure the protection of human health and the environment through the assessment of risks from the deliberate release or contained use of GMOs, it was felt that the protection of public health, through the accelerated dissemination of a COVID-19 vaccine, should prevail in this situation deemed unprecedented.
However, this temporary exemption is highly contested. Indeed, some expert groups have pointed out that this measure could be irresponsible because the development of vaccines based on GMO viruses could pose risks to human health and the environment, and these risks are not necessarily covered by general safety protocols designed to protect participants.[287]

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[285] https://www.consilium.europa.eu/en/press/press-releases/2020/07/14/vaccine-against-covid-19-council-adopts-measures-to-facilitate-swift-development/

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