microbiology research

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In Vitro Evaluation of the Antiviral Properties of Exogenous mRNA Encoding the Human MxA Protein

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Abstract: MxA is a cytoplasmic protein induced in human cells exposed to type I and III interferons. It can inhibit various viruses, including influenza A, by blocking the early steps of the viral replication cycle. The rapid advancement of mRNA-based technology has allowed us to evaluate the antiviral activity of MxA-mRNA, encoding intracellular MxA protein, and explore its potential as a therapeutic agent. In this study, we used in vitro transfection methods to obtain functional, mature MxA-mRNA and evaluate its activity within cells. We also observed an undesirable cellular response to transfection with exogenous mRNAs, which involved interferon III induction and reduced cell viability. Nevertheless, preventive administration of MxA-mRNA led to a specific 10–80-fold decrease in influenza A and B levels in cell supernatants—an effect not observed with the control GFP-Luc-mRNA. Additionally, we investigated the antiviral activity of MxA-mRNA against RNA viruses, such as SARS-CoV-2 and both serotypes of RSV, but we could not demonstrate a significant virus-specific effect of exogenous mRNA on their replication. We believe that mRNAs that encode native antiviral proteins have great therapeutic potential.

Keywords: exogenous mRNA; MxA; antiviral activity

Academic Editor: Caijun Sun

Received: 9 November 2024
Revised: 29 December 2024
Accepted: 22 January 2025
Published: 25 January 2025

Citation: Plotnikova, M.A.; Romanovskaya-Romanko, E.A.; Pulkina, A.A.; Shuklina, M.A.; Shurygina, A.-P.S.; Klotchenko, S.A. In Vitro Evaluation of the Antiviral Properties of Exogenous mRNA Encoding the Human MxA Protein. *Microbiol. Res.* **2025**, *16*, 32. https://doi.org/10.3390/microbiolres16020032

1. Introduction

Human myxovirus resistance protein A (MxA) is a 78 kDa cytoplasmic protein associated with the smooth endoplasmic reticulum [1]. IFN type I- and III-induced MxA is an important mediator of innate immunity, exhibiting antiviral activity against a wide range of RNA and DNA viruses [2,3]. Recent studies have demonstrated the antiviral activity of MxA against *Orthomyxoviridae* (influenza virus), *Bunyaviridae*, *Rhabdoviridae*, *Paramyxoviridae* (measles virus), *Picornaviridae*, *Togaviridae*, *Reoviridae*, *Hepadnaviridae* (HBV), and others [2,4].

While significant advances have been made in understanding the biochemistry and molecular biology of MxA, the precise mechanism by which MxA GTPase inhibits influenza A virus infection remains unknown [5,6]. MxA comprises a globular GTPase domain and an alpha-helical stalk, which are linked via a flexible bundle-signal element (BSE) consisting of three helices [7]. GTPase activity and oligomerization via the stalk and the BSE are required for its antiviral activity [7,8]. Within the stalk domain, MxA has a flexible loop, termed loop L4, and the unstructured loop L2 in the vicinity of L4 [9]. Various studies have demonstrated that L4 is a key determinant of antiviral specificity against influenza A and Thogoto virus [9–11]. In particular, amino acid variations in L4 (F561) can have significant consequences for altering MxA antiviral specificity [11].

The amino-terminal (N-terminal) region, which highly varies in length and sequence, is also an essential element that ensures the antiviral activity of MxA [9]. N-terminal region, and more precisely, leucine 41, is essential for the anti-influenza A virus (IAV) activity and correct subcellular localization of MxA.

To date, no cofactor that would be essential for MxA effector antiviral activity or correct subcellular localization has been identified [9]. In respiratory epithelial cells, MxA is thought to serve as an inflammasome sensor that recognizes the nucleoprotein (NP) of IAV, triggering a rapid inflammatory response [12]. In the NP of the 1918, pH1N1, and avian H5N1 strains, amino acid clusters were identified that confer resistance to human MxA. In particular, IAV strains carry amino acid variations in NP (D16, I/V100, P283, and Y313) for which the resistance to MxA has been described [6].

The antiviral properties of MxA make it a highly promising therapeutic agent. Pavlovic and colleagues demonstrated that the expression of cytoplasmic human MxA confers a high degree of resistance to influenza A virus and the rhabdovirus VSV in 3T3 cells, whereas sensitivity to the picornaviruses EMC and mengovirus, the togavirus SFV, and HSV-1 is not affected by MxA [13]. Stable translation of human MxA in murine 3T3 cells was achieved by plasmid DNA transfection.

In recent years, substantial progress has been made in understanding the structural features and metabolism of mRNA, as well as in developing methods for its delivery into eukaryotic cells. New technologies have made exogenous synthetic mRNA a more accessible tool, replacing protein-based formulations in cancer treatment, infectious disease therapy, and vaccination.

The concept behind using exogenous mRNA as a drug involves transferring a defined genetic message into the patient's cells with the ultimate goal of preventing or altering a particular disease state [14]. The proof of principle for a novel anti-inflammatory therapy was the nucleofection of IL-10 mRNA in monocytes and macrophages in a murine model of autoimmune myocarditis [15]. Earlier, Jirikowski and colleagues showed that the injection of a synthetic mRNA encoding vasopressin into the hypothalamus of vasopressin-deficient rats led to a temporary reversal of their diabetes insipidus [16].

Based on advancements in mRNA-based therapeutics technology, we assessed the therapeutic potential of exogenous mRNA encoding human MxA. Once translated from exogenous mRNA in the cell, the protein undergoes post-translational modifications resulting in a biologically active molecule that closely resembles its native structure. Here, we report that the MxA protein can be successfully translated within cells. Furthermore, we evaluate the inflammatory response to synthetic mRNAs and assess the antiviral effects of exogenous mRNA against influenza A and B viruses, respiratory syncytial virus (RSV), and SARS-CoV-2 in permissive cell models.

2. Materials and Methods

2.1. Synthesis of Exogenous mRNA by In Vitro Transcription

The plasmids contained a T7 promoter, followed by a 5′-UTR and 3′-UTR from the COVID-19 vaccine mRNA-1273 (Moderna, Cambridge, MA, USA) and a 114-nucleotide poly-A tail. The coding sequence (CDS) of the target sequence was synthesized by RT-PCR. The amino acid sequence of the MxA CDS fully aligned with the P20591 sequence (UniProt), including leucine 41 and phenylalanine 561. The only variation observed was a V → I substitution at position 379, which does not appear to be associated with the antiviral properties of MxA. As a negative control, we used a reporter construct containing green fluorescent protein (GFP) and luciferase (Luc) within a single open reading frame (ORF). GFP and Luc were separated by the P2A peptide from porcine teschovirus 1, which induces ribosome 'skipping'.

Plasmids were linearized with XbaI (New England Biolabs, Ipswich, MA, USA) overnight and purified using the Cleanup Mini kit (Evrogen, Moscow, Russia). Synthetic mRNAs were produced by in vitro transcription (IVT) using the HighYield T7 ARCA mRNA Synthesis Kit (#RNT-102, Jena Bioscience, Jena, Germany). Anti-Reverse Cap Analog (ARCA; #RNT-102, Jena Bioscience, Jena, Germany) was used for efficient translation of RNA. To maximize RNA yield and the fraction of capped transcripts, we used the ARCA/GTP ratio of 4:1. The reaction was performed according to the manufacturer's protocol. For modified RNAs, equimolar ratios of ATP and GTP were used alongside pseudouridine-5′-triphosphate (Ψ) and 5-methylcytidine-5′-triphosphate (m5C). Following RNA synthesis, the DNA template was removed by digestion with DNase Turbo (#AM1345,Thermo Fisher Scientific, Waltham, MA, USA). Afterward, synthetic mRNA was recovered by LiCl precipitation. RNA was diluted in RNase-free water, and the concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The RNA samples were analyzed using electrophoretic separation under denaturing conditions. Five hundred nanograms of RNA sample was mixed with an equal volume of RNA Gel Loading Dye (2×) (#R0641, Thermo Fisher Scientific, Waltham, MA, USA) and heated for 10 min at 70 °C. Samples were subsequently loaded into wells of 1% agarose gel (containing 0.5 μg/mL ethidium bromide) and run with 1× MOPS buffer (20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA) at room temperature.

2.2. Cell Lines

The A549 cells (ATCC, CCL-185) were maintained in F12K medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Vero (ATCC, CCL-81) and MDCK (FR-58) cell lines were maintained in AlphaMEM (Biolot, St. Petersburg, Russia) containing 10% fetal bovine serum (Biowest, Nuaillé, France). Cells were cultured at 37 °C, 5% CO_2 in a humidified atmosphere.

For transfection experiments, 1.2–2 × 10^4 cells/well were seeded overnight into 12-well plates before treatment with the obtained mRNAs. Before transfection, the growth medium was replaced with a serum-free medium (Gibco, Grand Island, NY, USA). RNA complexes were formed in the serum-free medium by mixing 0.3 μL of GenJector-U reagent (Molecta, Moscow, Russia) and 150 ng exogenous mRNA in a 10 μL volume per well of a 96-well plate. To study toxicity potentially caused by the carrier, the Lipofectamine™ MessengerMAX Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used along with the GenJector-U reagent (Molecta, Moscow, Russia). RNA complexes were prepared strictly in accordance with the manufacturer's instructions, using 0.3 μL of the reagent for 150 ng of RNA in a 10 μL volume.

2.3. ELISA and Immunofluorescence Staining

Human MxA concentrations in cell lysates were measured using the MxA Protein Human ELISA kit with supplied standards (#RD194349220R, BioVendor, Brno, Czech Republic). Optical densities were measured at 450 nm (OD_{450}) on a CLARIOstar plate photometer (BMG LABTECH, Ortenberg, Germany).

For immunofluorescence staining, cells in monolayer were fixed in 5% formaldehyde and permeabilized. After washing with a 1× PBST solution (0.05% Tween 20), wells were blocked with a 5% Blotting-Grade Blocker (Bio-Rad, Hercules, CA, USA). An immunofluorescence assay was performed with 100 μL of biotinylated mouse monoclonal MxA/Mx1 antibody (Novus Biologicals, Centennial, CO, USA), followed by Streptavidin-Cy5 detection (Thermo Fisher Scientific). Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize F-actin in cells. Cell nuclei were stained with

DAPI (AppliChem, Darmstadt, Germany). Images were captured with a Leica TCS SP8 inverted confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.4. Cell Viability Assessment

For the MTT assay, 20,000 transfected cells per well were plated in a 96-well plate and incubated at 37 °C, 5% CO_2. At the indicated time points, a 10% volume of MTT solution in DPBS (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated at 37 °C for 4 h. Then, 200 µL of dimethylsulfoxide was added to dissolve formazan crystals after removing the medium. Optical density (OD) was measured at 490 nm wavelength. To calculate cell viability, three biological replicates were used, with the average OD_{490} value from intact cells set as 100%.

For annexin Yo-Pro/PI staining, transfected cells were harvested by trypsinization. Then, the cells were resuspended in 100 µL of PBS and incubated with 5 µL of Yo-Pro-FITC (fluorescein isothiocyanate) solution and 5 µL of propidum iodide for 15 min at room temperature in the dark. Flow cytometry was used to measure cell apoptosis. Cell viability was assessed following the detailed protocol described in [17].

2.5. RT-PCR Analysis

Total RNA was isolated from A549 cells using TRIzol (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentrations and integrity were analyzed using a NanoDrop.

One microgram of total RNA was treated with DNase (Promega, Madison, WI, USA) and then directly reverse-transcribed using $oligo(dT)_{16}$ primers and the RNAscribe RT kit (Biolabmix, Novosibirsk, Russia). Complementary DNA synthesis was carried out at 50 °C for 50 min. The enzyme was inactivated at 80 °C for 5 min. Products were diluted (1:2) and stored at −20 °C until use.

Real-time PCR assays were performed using a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). qPCR was performed in 25 µL final reaction volumes containing 12.5 µL of the BioMaster HS-qPCR 2× mix (Biolabmix, Novosibirsk, Russia), previously developed primers [18], and 2 µL of cDNA.

2.6. Antiviral Activity

To study the antiviral effect of the designed mRNAs, only the 'preventive' treatment protocol was used. MDCK or Vero cells were transfected with 150 ng of mRNA and then incubated with viruses for 18–20 h.

All viral strains were obtained from the Virus and Cell Culture Collection of the Smorodintsev Research Institute of Influenza (St. Petersburg, Russia).

Influenza viruses were grown in 11-day-old embryonated eggs and stored at −80 °C. The infectivity of the viruses in MDCK cells was 1.7×10^8, 2.2×10^8, and 2.4×10^6 $TCID_{50}$/mL for A/Puerto Rico/8/1934 (H1N1), A/Singapore/INFIMH-16-0019/2016 (H3N2), and B/Malaysia/2506/04 (Vic), respectively. Virus titration in the presence of a single dose of mRNA (150 ng per well of a 96-well plate) was used to assess the virus-inhibitory effect of MxA-mRNA. Twenty-four hours after transfection, MDCK cells were infected with 10-fold dilutions of IAV. Viral titers in culture supernatants were determined by hemagglutination assay (HA).

SARS-CoV-2 virus hCoV-19/Russia/SPE-RII-3524V/2020 (GISAID ID EPI_ISL_415710) was isolated from a patient's oropharyngeal swab and cultured in Vero cells (ATCC CCL-81). The infectivity of the SARS-CoV-2 virus stock in Vero cells was 1.26×10^7 $TCID_{50}$/mL. To assess antiviral activity, Vero cells pre-transfected with mRNAs were infected with the virus at doses of 1, 0.1, 0.01, and 0.001 moi. Viral production in supernatants collected 8 and 24 h after infection was evaluated using titration in Vero cells. Viral infectious activity in

response to mRNA transfection was calculated using the Reed—Muench method [19]. The infection was considered productive when visible CPE was observed. All manipulations with SARS-CoV-2 were performed in a BSL-3 facility.

One- to four-fold dilutions of A2 and B9320 RSV serotypes were also used to evaluate the antiviral activity of the mRNA. RSV viral load was measured at 24 h post-infection by in situ cellular ELISA with anti-F antibodies. For ELISA, cell supernatants were removed 24 h post-infection, and the cells were fixed with acetone, blocked, and incubated with custom diagnostic mouse monoclonal antibodies 4F2 [20]. Detection was performed using goat anti-mouse horseradish peroxidase (GAM-HRP, Bio-Rad).

2.7. Statistical Data Processing

Data processing was carried out in Microsoft Excel. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). The nonparametric Kruskal-Wallis test with Dunn's multiple comparisons was used to identify statistically significant differences between groups.

3. Results

3.1. Generating Functional mRNA

The full-length human MxA encoding sequence was cloned downstream of the T7 promoter. The mRNA was obtained by in vitro transcription (Figure 1A) and contained a cap (ARCA), 5′ and 3′ UTRs of 57 and 110 nucleotides (nt), modified pseudouridine-5′-triphosphate (Ψ) and 5-methylcytidine-5′-triphosphate (m5C) bases, and a 114 nt template-encoded poly-A tail.

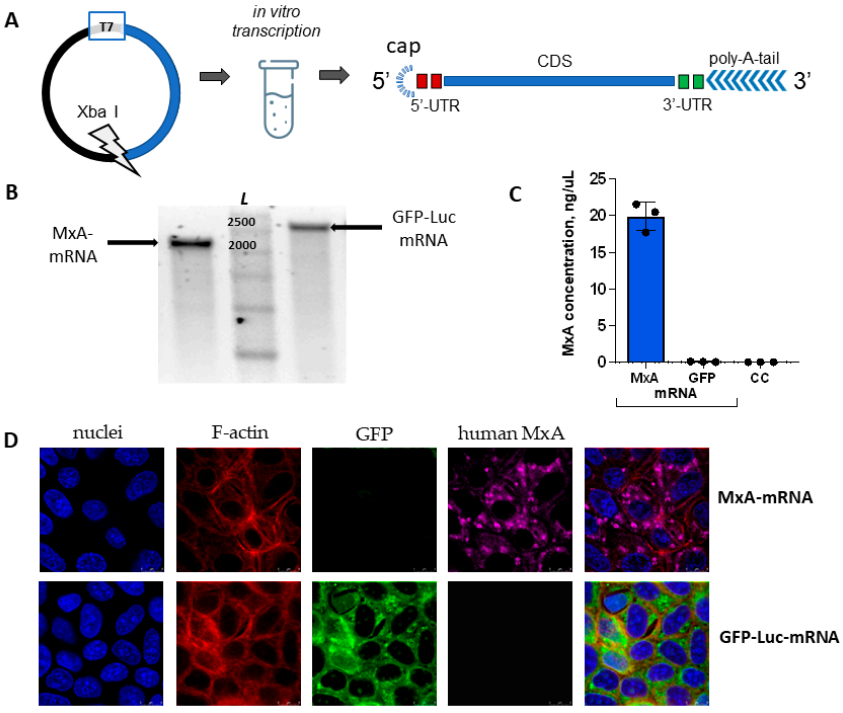

Figure 1. Design and translation of exogenous mRNA encoding human MxA. (**A**) Structure of MxA-mRNA was designed as described in Methods. (**B**) Agarose gel electrophoresis of 2300-nt MxA-mRNA and 2800-nt GFP-Luc-mRNA. L marks the molecular weight ladder AM1750 (Thermo). (**C**) MxA concentration in MDCK cell lysates 24 h after transfection with exogenous mRNAs, measured by ELISA. CC—control cells that were transfected with the GenJector-U carrier but did not contain exogenous mRNA. (**D**) Fluorescent microscopy of MDCK cells transfected with MxA-mRNA (top row) and GFP-Luc-mRNA (bottom row); stained with DAPI for nuclei (blue), Alexa Fluor 488 Phalloidin for F-actin (red), and Cy5-antibody for MxA (magenta).

UTRs of messenger RNAs are known to form secondary structures which are important for correct ribosome scanning and dissociation, rate of translation, and the half-life of mRNA in cells [21]. In native human RNA, the median length of 5′ UTRs ranges between 53 and 218 nucleotides, while 3′ UTRs are highly heterogenous, with a median length of about 1000 nucleotides [22]. When designing exogenous mRNA, it is important to aim for the shortest UTRs possible to reduce the risk of aberrant transcripts and translation.

Another factor that contributes to the increased stability and translational capacity of mRNA is the use of naturally occurring modified nucleosides. The study by Karikó showed that mRNAs with Ψ modification have a higher translational capacity than those without modification in all tested mammalian systems [23]. Furthermore, modified bases can help significantly reduce the immunogenicity of exogenous mRNAs in vivo [24].

The structure of the 5′ cap also enables discrimination between self and non-self mRNA molecules and affects immunogenicity [25]. To mimic fully processed mRNAs and avoid stimulation of innate immunity, it is advisable to create synthetic mRNAs with modified cap analogs. Our mRNA was capped during transcription by adding the anti-reverse cap analog (ARCA), in which the hydroxyl group closest to m7G is replaced by a methoxy group. As shown previously, the translational efficiency of ARCA-capped transcripts in a rabbit reticulocyte lysate is 2.3- to 2.6-fold higher than that of m7 GpppG-capped transcripts [26]. However, it should be noted that there are now synthetic cap analogs with even greater advantages, such as CleanCap.

As Figure 1B illustrates, the mature mRNA encoding MxA (MxA-mRNA) is approximately 2300 nt long. For visualization and as a negative control, we also synthesized mRNA encoding the GFP and Luc proteins in a single ORF, separated by the P2A peptide (GFP-Luc-mRNA). GFP-Luc-mRNA has a length of 2800 nt, comparable to MxA-mRNA, which may be important for validating delivery. Like MxA, the GFP and Luc proteins have intracellular localization. In mammalian cells, mRNAs containing 100–250 adenosine residues at the 3′-end are translated with the highest efficiency [14]. Thus, our MxA-mRNA contained all the necessary elements for its efficient translation in cell models.

To estimate the concentration of MxA protein produced by the transfected cells, we performed ELISA on whole-cell extracts (Figure 1C). For transfection, we chose the MDCK cell line which is highly permissive to influenza virus. Twenty-four hours after transfection with 100 ng of exogenous MxA-mRNA, the production of the human MxA protein reached 20 ng/μL. No proteins highly homologous to human MxA were detected in the negative cell control or following GFP-Luc-mRNA transfection. The translational activity of MxA-mRNA was also analyzed in A549, Vero, and HEp-2 cell lines. In similar experimental conditions, the level of translated MxA in all cell lysates was at least 15 ng/mL.

In a further set of experiments, we tested the intracellular distribution of the MxA translated from exogenous MxA-mRNA (Figure 1D). MDCK cells were transfected with MxA-mRNA (100 ng per 10^5 cells), and the MxA localization was analyzed by immunofluorescence using MxA-specific monoclonal antibodies.

3.2. Immunogenicity

Immunogenicity is a well-known and significant challenge in the development of therapeutic agents based on exogenous mRNA [23,27,28]. It is of particular importance when using exogenous mRNAs as antiviral agents. On one hand, the protective effect on viral pathogenesis may result from the enhanced activation of the innate immune system and the release of type I and III interferons. On the other hand, increased immunogenicity of exogenous mRNAs may reduce translation efficiency and induce an undesirable inflammatory response [29].

We used flow cytometry to assess the number of live (L), dead (D), apoptotic (A), and necrotic (N) cells 24 h after the transfection of MDCK cells with exogenous mRNA (Figure 2A). For transfection, 150 ng of mRNA and 0.3 µL of the transfection reagent GenJector-U reagent (Molecta, Russia) were used. As shown in Figure 2B, mRNA transfection resulted in a slight decrease in viable cell counts, from 85% in the control condition to 79–80% for both MxA-mRNA and GFP-Luc-mRNA. Moreover, the transfected cells tended to exhibit higher percentages of necrotic and apoptotic cells. The decrease in cell viability induced by exogenous mRNA was further analyzed with a colorimetric MTT assay. Using this method, we were also able to detect a decrease in cell viability 24 h after mRNA transfection (Figure 2C).

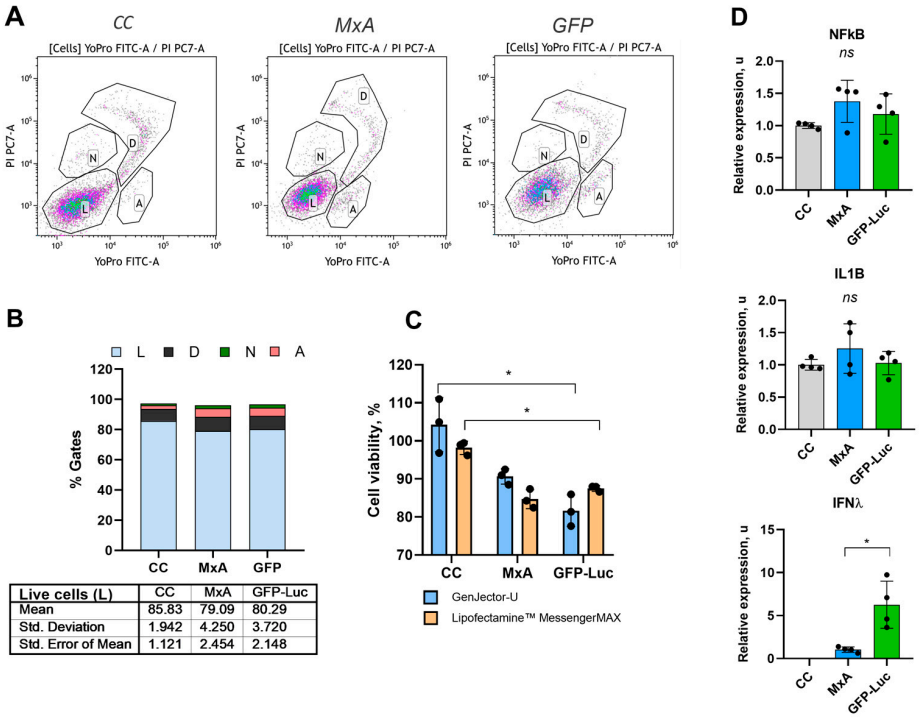

Figure 2. Immunogenic and interferon-inducing properties of exogenous mRNAs. (**A**) Detection of apoptosis in MDCK cells using YO-PRO-1. Live cells (L) are negative for both YO-PRO-1 and PI. Cells in early apoptosis (A) show increased YO-PRO-1 fluorescence but remain PI-negative. Dead cells (D) are YO-PRO-1 and PI-bright, but the fluorescence of both dyes reduces as DNA degrades when cells transition to necrosis (N) [17]. (**B**) Detection of apoptosis in MDCK cells using flow cytometry. Bars represent L, D, A, and N populations averaged from three independent replicates. (**C**) Graph of cell viability percentages measured by the MTT assay in MDCK cells 24 h after mRNA transfection. (**D**) mRNA expression levels in A549 cells 24 h after transfection CC—control cells that were transfected with the GenJector-U carrier (or with Lipofectamine™ MessengerMAX for (**C**)) but did not contain exogenous mRNA. MxA—cells transfected with MxA-mRNA. GFP—cells transfected with GFP-Luc-mRNA. *—$p < 0.05$; ns—no significant difference.

To assess potential toxicity from the carrier, Lipofectamine™ MessengerMAX was used along with the GenJector-U reagent (Molecta, Russia). The MTT cell viability assay was also performed in Vero and A549 cells. Viable cell counts for both MxA-mRNA and GFP-Luc-mRNA were reduced by approximately 10–15% across all studied cell lines, regardless of the transfection reagent used.

Toll-like receptor (TLR) signaling can trigger cell apoptosis through multiple mechanisms. In vitro, transcribed RNAs are recognized by cytoplasmic and endosomal RNA sensors, which include TLR3, TLR7, TLR8, RIG-I, MDA5, NOD2, IFIT-2, DDX60, DHX9, DDX3, the DDX1-DDX21-DHX36 complex, HMGB proteins, and LRRFIP1, and others [30].

Activation of TLR signaling may involve the NF-κB and IL-1b metabolic pathways and result in the release of type I interferon.

As measuring the expression of these genes in MDCK cells proved challenging due to a limited access to reagents, we evaluated gene expression in A549 cells, which have also shown decreased viability after transfection with MxA-mRNA. Using RT-qPCR, we evaluated the expression levels of NFkB, IL1B, and IFNλ in A549 cells following the transfection with our exogenous mRNAs. As shown in Figure 2D, the expression levels of the transcription factor NFkB and the downregulated inflammatory cytokine IL1B remained virtually unchanged after mRNA transfection. However, both MxA-mRNA and GFP-Luc-mRNA induced IFNλ expression. IFNλ has broad antiviral activity against RNA viruses [31,32]. Notably, GFP-Luc-mRNA triggered a statistically significant 6-fold increase in IFNλ expression compared to MxA-mRNA. The induction of IFNλ expression suggests a potential mechanism that underlies the enhanced non-specific antiviral response associated with GFP-Luc-mRNA.

3.3. Antiviral Activity of MxA-mRNA

To assess the antiviral effect of MxA-mRNA, we infected monolayer permissive cell cultures with RNA viruses. We used the MDCK cell line to demonstrate the antiviral potential of MxA-mRNA against influenza viruses. Cells were inoculated with viruses at various concentrations 18–20 h after transfection with either MxA-mRNA or GFP-Luc-mRNA.

As shown in Figure 3A, MxA-mRNA caused a weak yet statistically significant reduction in viral titers of two influenza A and influenza B virus subtypes at 24 h post-infection. Regardless of the influenza virus strain, transfection with MxA-mRNA resulted in an approximately 10- to 80-fold decrease in hemagglutinating virions in cell supernatants. It is worth noting that a trend toward a lower viral load in response to GFP-Luc-mRNA transfection was also observed for the H1N1 and B/Vic strains. We suggest that this may be due to the ability of our exogenous mRNAs to induce interferon, as discussed above. However, in the case of H3N2, pre-transfection with GFP-Luc-mRNA led to a small increase in virus yield.

Cell lines constitutively producing cytoplasmic human MxA were previously shown to be highly resistant to fowl plague virus (also known as avian influenza). The viral titers in the 24-h supernatants of the modified cell lines were between 100- and 200-fold lower than those of the control cells [13]. In our experiments, mRNAs demonstrated a significantly weaker effect, which can be attributed to both their immunogenicity and strain specificity. Avian influenza viruses were found to be generally more sensitive to MxA than human strains [4]. Another study reported that human influenza strains can acquire adaptive mutations in NP to evade MxA restriction [6]. In particular, the NP of the 1918 ('Spanish' H1N1) and 2009 (H1N1pdm09) influenza A virus strains has four amino acids that seem to reduce the sensitivity of these strains to the effects of MxA.

We aligned the amino acid sequences of the NP proteins of both IAV strains used in our study. It has been reported [6] that MxA resistance-associated amino acids—D16, I/V100, P283, and Y313—are commonly found in classical seasonal isolates of human influenza virus at very high frequencies (>98%). Both our IAV strains carried D16, V100, P283, and Y313, so the difference in inhibition is not sufficiently explained by the presence of substitutions in the NP amino acid sequence. It is more likely that the initial virus titers play a more substantial role.

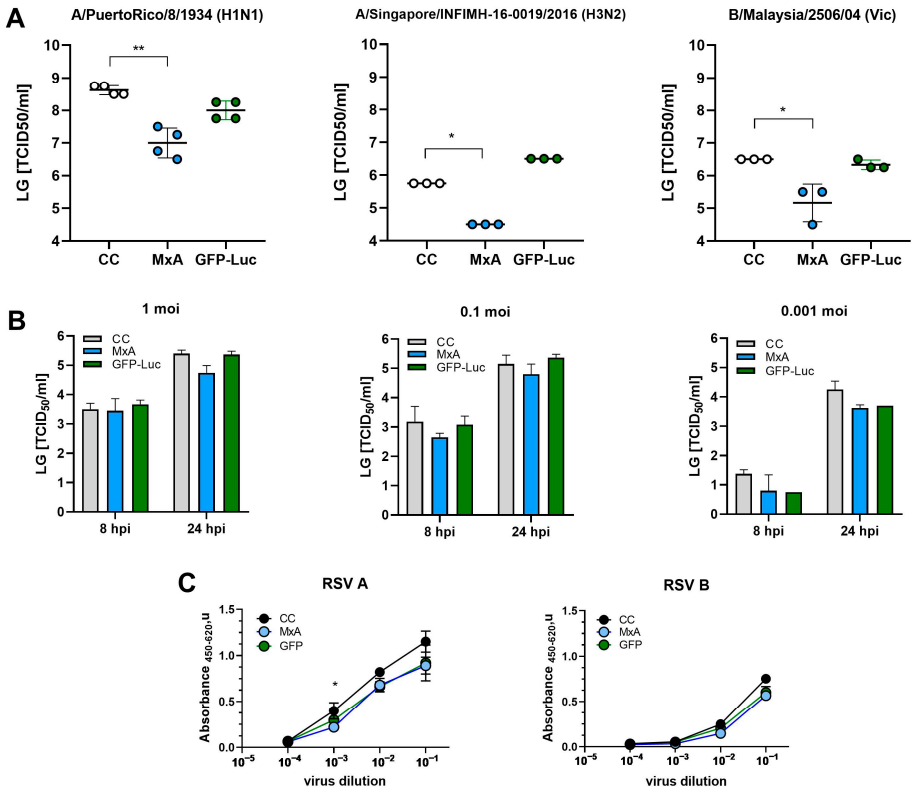

Figure 3. Antiviral activity of MxA-mRNA in vitro. (**A**) $TCID_{50}$ values measured 24 h after preventive treatment of MDCK cells with MxA-mRNA (hemagglutination assay). (**B**) SARS-CoV-2 viral load in Vero cells pre-transfected with mRNAs. Cells were infected with 1, 0.1, or 0.001 moi the hCoV-19/Russia/SPE-RII-3524V/2020 strain. (**C**) Cell-based ELISA against RSV F-protein in Vero cells transfected with exogenous mRNAs. Viral loads were determined 24 h post-infection and additionally at 8 h for SARS-CoV-2. The data are presented as means with SD. *p*-values for statistical significance were determined by comparing groups using the Kruskal-Wallis test: *—$p < 0.05$; **—$p < 0.001$. CC—control cells that were transfected with the GenJector-U carrier but did not contain exogenous mRNA. MxA—cells transfected with MxA-mRNA. GFP—cells transfected with GFP-Luc-mRNA.

Next, we tested the sensitivity of SARS-CoV-2 to exogenous MxA-mRNA. Our results show that preventive administration of MxA-mRNA does not lead to a statistically significant decrease in SARS-CoV-2 $TCID_{50}$ neither 8 nor 24 h after infection with different doses of the virus (not all doses are shown). As seen in Figure 3B, a slight trend toward decreased viral load is observed for both exogenous mRNAs. The current literature indicates that MxA expression is significantly higher in COVID-19 patients than in the non-COVID-19 group [33]. It has also been suggested that drugs enhancing MxA expression have great therapeutic potential against SARS-CoV-2 [34,35]. Yet, our study failed to demonstrate encouraging results regarding the antiviral activity of MxA-mRNA against SARS-CoV-2.

As the final stage of our study, we also examined the sensitivity of RSV A2 and RSV B 9320 strains to MxA-mRNA transfected into Vero cells. Similar to SARS-CoV-2, MxA-mRNA showed a rather weak antiviral effect against both RSV serotypes. A statistically significant reduction in viral load was observed only when cells were infected with RSV A at a dose of 0.1 moi. Similar results were obtained in 1999 in a study of virus yield produced by RSV A2 in MxA-expressing cell lines compared to cells lacking the MxA protein [36].

4. Discussion

Exogenous mRNA has emerged as a rapidly advancing technology with significant potential in vaccinology and therapeutics. mRNA-based drugs offer transient and controlled expression of a protein with a desired conformation in cells while avoiding genomic

integration. In this study, we assessed the possibility of translating an intracellular cytoplasmic protein MxA from exogenous mRNA and evaluated its functionality in cell cultures. MxA is induced by the interferon system and plays an important role in protecting cells from viral infection. Therefore, exploring the antiviral potential of mRNA encoding MxA is extremely relevant.

We demonstrated that after transfection with MxA-mRNA, cells produced up to 20 ng/mL of MxA protein, as detected by specific antibodies. The viability of cells transfected with exogenous mRNAs was found to decrease by 10–15% compared to intact cells. Assuming that the MxA-mRNA and GFP-Luc-RNA we designed could be immunogenic, we assessed the expression of NFkB, IL1B, and IFNλ in cells following transfection. Both mRNAs were found to induce interferon production.

Finally, the study demonstrated that MxA-mRNA suppressed the replication of influenza A and B viruses by 10–80 times, while GFP-Luc-RNA showed no non-specific antiviral activity. MxA-mRNA also had no detectable inhibitory effect on either SARS-CoV-2 or RSV A and B. Despite its limitations, we consider MxA-mRNA to be a promising therapeutic agent. Its antiviral effect could potentially be enhanced by reducing its immunogenicity.

Author Contributions: Conceptualization, M.A.P., E.A.R.-R., A.-P.S.S. and S.A.K.; methodology, M.A.P., E.A.R.-R., A.A.P., M.A.S., A.-P.S.S. and S.A.K.; software, M.A.P. and M.A.S.; validation, M.A.P.; formal analysis, M.A.P.; investigation, M.A.P., E.A.R.-R., A.A.P., M.A.S. and S.A.K.; resources, S.A.K.; data curation, M.A.P. and S.A.K.; writing—original draft preparation, M.A.P.; writing—review and editing, M.A.P. and S.A.K.; visualization, M.A.P.; supervision, M.A.P. and S.A.K.; project administration, S.A.K.; funding acquisition, M.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Russian Science Foundation, grant No. 23-25-00433, project title: "Assessment of antiviral effect of the mRNA encoding human MxA protein" (supervisor M.A. Plotnikova), https://rscf.ru/project/23-25-00433/ (accessed on 21 January 2025).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data might be made available upon request to the corresponding author.

Acknowledgments: We gratefully acknowledge all of the people who have contributed to this paper.

Conflicts of Interest: The authors declare no conflicts of interest.

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