Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 158 (2009) 93–99

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Gene optimization leads to robust expression of human respiratory syncytial virus nucleoprotein and phosphoprotein in human cells and induction of humoral immunity in mice

Fernando M. Simabuco, Rodrigo E. Tamura, Cassiano Carromeu, Luis E. Farinha-Arcieri, Armando M. Ventura*

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil

Article history:
Received 30 October 2008
Received in revised form 21 January 2009
Accepted 27 January 2009
Available online 4 February 2009

Keywords:
Human respiratory syncytial virus
Nucleoprotein
Phosphoprotein
DNA vaccine
Gene optimization

A B S T R A C T

Human respiratory syncytial virus (HRSV) is the major pathogen leading to respiratory disease in infants and neonates worldwide. An effective vaccine has not yet been developed against this virus, despite considerable efforts in basic and clinical research. HRSV replication is independent of the nuclear RNA processing constraints, since the virus genes are adapted to the cytoplasmic transcription, a process performed by the viral RNA-dependent RNA polymerase. This study shows that meaningful nuclear RNA polymerase II dependent expression of the HRSV nucleoprotein (N) and phosphoprotein (P) proteins can only be achieved with the optimization of their genes, and that the intracellular localization of N and P proteins changes when they are expressed out of the virus replication context. Immunization tests performed in mice resulted in the induction of humoral immunity using the optimized genes. This result was not observed for the non-optimized genes. In conclusion, optimization is a valuable tool for improving expression of HRSV genes in DNA vaccines.

1. Introduction

The human respiratory syncytial virus (HRSV) is a negative-sense genome single-stranded RNA virus that belongs to the *Paramyxoviridae* family and to the *Pneumovirinae* subfamily (Collins et al., 2001). HRSV is considered the major pathogen causing respiratory disease in infants and neonates worldwide, which may result in serious complications such as pneumonia and bronchiolitis (Holberg et al., 1991). This virus is responsible for 38% of the lower respiratory tract infections in infants up to 1 year of age. Half of these infants have re-infections after 1 year (Schmidt et al., 2004). HRSV is also a significant cause of respiratory disease in the elderly (Han et al., 1999), immune-compromised patients, such as bone marrow transplant patients (Hall et al., 1986), and is related to the development of asthma in childhood (Lemanske, 2004).

HRSV virion is enveloped and carries a genome of about 15,000 nucleotides that encodes 11 proteins (Collins et al., 2001). The nucleoprotein or N protein associates with the viral RNA, forming a helical structure and guaranteeing resistance to RNases (Meric et al., 1994). The phosphoprotein or P protein has two known functions: it interacts with the N protein, giving specificity for viral RNA encapsidation (Curran et al., 1995; Spehner et al., 1997); and interacts with the L protein (the major unit of the virus replication complex), conferring stability and the correct placement in the ribonucleocomplex for RNA synthesis (Bowman et al., 1999; Horikami et al., 1992). The genome encodes also three transmembrane surface proteins (F, G, SH), a matrix protein (M), a nucleocapsid-associated protein (M2-1), an M2-2 protein (the second product of the M2 gene) and two nonstructural proteins (NS1, NS2) (Collins et al., 2001).

The most effective treatment against HRSV is a humanized anti-F monoclonal antibody that inhibits viral attachment to the cell surface. Its administration is a recommended preventative measure for high risk groups such as premature neonates, although large-scale usage is very limited due to a poor cost-effectiveness ratio (Joffe et al., 1999). A formalin-inactivated vaccine was tested in humans, but no protection resulted and a vaccine-enhanced disease was observed (Kim et al., 1969; Fulginiti et al., 1969).

Promising results were obtained using DNA vaccines against the bovine respiratory syncytial virus (BRSV) F protein in calves (Taylor et al., 2005); and against HRSV F and N proteins in chimpanzees (Vaughan et al., 2005). However, a recent study showed that the RNA polymerase II dependent expression of HRSV F protein can only be achieved at high levels with the optimization of the gene, which involves the elimination of premature polyadenylation sites (Ternette et al., 2007a). In a further study, the same group used the

* Corresponding author at: Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Professor Lineu Prestes, 1374, São Paulo, SP, 05508-900, Brazil. Tel.: +55 11 3091 7276; fax: +55 11 3091 7354.
E-mail address: amventur@icb.usp.br (A.M. Ventura).

0166-0934/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2009.01.024

optimized F gene to test a DNA vaccine in mice, showing induction of antibodies and protection against HRSV (Ternette et al., 2007b). In another recent study, DNA vaccines containing the optimized BRSV N or F genes were used to induce robust cell-mediated immunity and protection of calves against the BRSV challenge (Boxus et al., 2007).

In the present study, HRSV (strain A2) optimized N and P genes were synthesized and cloned in a mammalian expression plasmid under the control of a cytomegalovirus immediate early (CMVie) promoter, transcribed by RNA polymerase II. After transfection of human cells, protein expression was monitored by Western blot and immunofluorescence assays for optimized and non-optimized genes. In both experiments, neither the N protein nor the P protein could be detected for plasmids containing the natural sequence of the genes. However, robust expression of both proteins was obtained after optimization. The study shows that these proteins are expressed in different intracellular localizations compared with HRSV infected cells. Immunization tests were performed and only the optimized genes were able to generate humoral immunity. The positive effect of HRSV N and P gene optimization is described as an approach that might be considered for the development of a DNA vaccine against HRSV.

2. Material and methods

2.1. Gene optimization

HRSV (strain A2) N and P gene coding sequences (respectively, AAC14896 and AAC14897) were submitted to GeneArt (Regensburg, Germany) and optimized using the GeneOtimizer software, which optimizes the codon usage and the GC content and eliminates polyadenylation sites, splicing sites, killer motifs and RNA secondary structures. Afterwards, the genes were synthesized, sequenced and cloned in a standard GeneArt plasmid. Analyses of the codon adaptation index (CAI) of the genes were made using the program provided by Evolving Code Group (http://www.evolvingcode.net/codon/cai/cai.php). Analyses of premature polyadenylation sites were made using the program provided by the National Research Council, Institute of Biomedical Technologies (http://zeus2.itb.cnr.it/~webgene/wwwHC_polya.html).

2.2. Cloning

The HRSV (strain A2) N and P non-optimized genes were amplified by reverse transcription and polymerase chain reaction (RT-PCR). Briefly, HEp-2 cells were infected with HRSV strain A2 and total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, USA). cDNA was generated using random primers and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Primers were designed with restriction endonuclease sites flanking the genes for subcloning into pShuttle (Clontech, Mountain View, USA). The 5′–3′ primer sequences are presented below with the restriction sites underlined:

Ns: TAGCTAGCGAAGATGGGGCAAATACAACC (NheI)
Nas: CCCTTAAGTTAACTCAAAGCTCTACATCATTATC (AflII)
Ps: TAGCTAGCCAAATAAATCATCATGGAAAAG (NheI)
Pas: CCCTTAAGGTTGGATGATTGGGTTGGTTAG (AflII)

Following PCR amplification, the products were cloned into pCR-4-TOPO (Invitrogen) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, California, USA) and the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Finally, the N and P genes were excised from pCR-4-TOPO and sub-cloned into pShuttle DNA vectors, generating pN and pP, respectively. The N and P optimized genes, synthesized by GeneArt, were also designed with specific restriction endonuclease sites (XbaI and AflII) to allow for the sub-cloning into pShuttle, generating pNopt and pPopt, respectively.

2.3. DNA plasmid transfection and HRSV infection

For transfection of 6×10^4 HEp-2 or HEK293 cells, 3 μl of polyethyleneimine (1 mg/ml) were added for each μg of DNA in 150 mM NaCl, incubated for 20 min and poured over the cells containing modified Eagle's medium (MEM) with 10% of fetal bovine serum (FBS). For infection, cells were incubated with HRSV (strain A2) at a multiplicity of infection (MOI) of 100. The HRSV was purified by polyethylene glycol (PEG) precipitation as described by Trépanier et al. (1981). The virus was titrated by the end-point method in HEp-2 cells, which consisted of diluting the virus solution and observing the last dilution that presented a cytopathic effect after 48 h.

2.4. Western blot

After DNA transfection or HRSV infection, cells were washed with phosphate buffered saline (PBS), scraped and lysed with a SDS sample buffer (50 mM Tris–HCl, pH 6.8; 10 mM EDTA; 10% glycerol; 0.2% bromphenol blue; 2% SDS; 1% β-mercaptoethanol). The protein extracts were separated by SDS-PAGE, transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, United Kingdom), which was incubated with a blocking buffer (0.1% Tween and 5% non-fat dry milk in PBS), and then with anti-N and anti-P polyclonal antibodies previously obtained (Simabuco et al., 2007). The membrane was washed three times with washing solution (Tween 0.1% in PBS) and incubated with an anti-mouse peroxidase-conjugated antibody (KPL, Washington, USA). After three washes with washing solution, the membrane was treated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, USA), exposed to Hyperfilm (Amersham) and visualized. A control Western blot, using an anti-β tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, USA), was performed under the same conditions.

2.5. Immunofluorescence

HEp-2 cells were cultivated in a 16-well chamber slide (Nunc, Rochester, USA). After plasmid DNA transfection or HRSV infection, they were washed with PBS, fixed with 4% paraformaldehyde, washed again and permeabilized with 0.5% Triton X-100 (Sigma, St Louis, USA). The cells were incubated with monoclonal antibodies diluted in PBS containing 0.2% Triton and 3% non-fat dry milk against N (B37) and P (C771) proteins, provided by Dr. Erling Norrby (Orvell et al., 1987). Cells were washed with washing solution (Triton 0.2% in PBS) and incubated with an anti-mouse FITC-conjugated antibody (KPL) diluted in PBS containing 0.2% Triton and 3% non-fat dry milk. Finally, cells were washed with washing solution, incubated with DAPI (4′,6-diamidino-2-phenylindole) solution for 10 min, washed again and analyzed with a fluorescence microscope (Carl Zeiss, Thornwood, USA).

2.6. Separation of the nucleus and cytoplasm extracts

The CelLytic NuCLEAR Extraction Kit (Sigma) was used, following the manufacturer instructions. Briefly, the cells were washed with PBS and treated with the kit lyses buffer containing IGEPAL CA-630 at a final concentration of 0.6%. The supernatants, which consisted of the cytoplasm protein extracts, were collected. The

Table 1
Optimization of the HRSV N and P gene sequences.

	Number of premature poliadenilation consensus sequences (nucleotide positions in the gene)	Codon adaptation index (CAI)
N	14 (37, 65, 133, 175, 237, 443, 477, 501, 505, 559, 798, 799, 805, 1086)	0.635
Nopt	0	0.979
P	7 (52, 69, 135, 195, 366, 388, 422)	0.696
Popt	0	0.969

nuclei were washed with PBS, and nuclear protein extracts were collected with extraction buffer (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 0.42 M NaCl; 0.2 mM EDTA; 25% (v/v) glycerol).

2.7. Immunization tests

Eight-week-old female BALB/c mice were purchased from the Isogenic Mice Facility of the Biomedical Sciences Institute, University of Sao Paulo. All animal procedures were approved by the local ethical committee for animal care. Before inoculations and blood extractions, all mice were anesthetized with xylazine (0.4 mg per animal) and ketamine (3 mg per animal) administered intraperitoneally. Blood samples were collected through the retro-orbital plexus, left at 37 °C for 15 min, at 4 °C for 15 min and centrifuged at 5000 × *g* for 5 min. The sera were collected and stored at −20 °C until further use. Plasmids were purified by cesium chloride gradients and DNA inoculations were performed via intramuscular injection of 50 μl (50 μg) of the DNA solution in each quadriceps (Sasaki et al., 2003). Three weeks after the first dose, blood samples were collected and the second dose was applied. After 3 weeks, blood samples were collected again. In a second immunization experiment, aluminum phosphate was added to the DNA solution at a final concentration of 450 μg/ml (Ulmer et al., 1999).

2.8. Enzyme immunoassay (EIA)

The plates (96-well) were coated with 200 ng/well of N or P fused to maltose binding protein, purified as previously described (Simabuco et al., 2007). The plates were then blocked with PBS containing 0.05% Tween and 10% non-fat dry milk and incubated with immunized mice sera in serial dilutions. The plates were washed with washing solution (PBS containing 0.05% Tween) and incubated with an anti-mouse peroxidase-conjugated antibody (KPL). The plates were washed again with washing solution, incubated with ortho-phenyldiamine (Sigma) solution and H_2O_2. Absorbances at 492 nm were measured.

3. Results

3.1. Analysis of N and P expression by Western blot

The process of optimization was carried out by GeneArt for the HRSV (strain A2) N and P genes. Codon usage and the GC content were optimized; premature polyadenylation sites, splicing sites, killer motifs and unwanted RNA secondary structures were eliminated. The optimized genes were synthesized and sent cloned in standard plasmids. The optimized genes were then sub-cloned into pShuttle, generating pNopt and pPopt. The non-optimized genes were cloned into the same plasmid, generating pN and pP. The codon adaptation indexes (CAI) improved from 0.635 to 0.979 for the N gene and from 0.696 to 0.969 for the P gene after the optimization process (Table 1). The premature polyadenylation sites before the optimization were 14 for the N gene and 7 for the P gene. Both were reduced to zero after the optimization process (Table 1).

HEK293 cells were transfected with pN, pP, pNopt, pPopt and the empty plasmid. After 72 h, Western blots were performed with the protein extracts of each transfection as well as positive extracts from HEp-2 cells infected with HRSV. As seen in Fig. 1 (top panels), no protein was detected in extracts from cells transfected with pN (panel A) and pP (panel B) containing the non-optimized genes. However, DNA pNopt (panel A) and pPopt (panel B) vectors containing the optimized genes, showed robust expression of the respective proteins, presenting the expected molecular weights (42 kDa for N protein and 33 kDa for P protein), as observed for the proteins expressed in HRSV infected cells. A control Western blot using an anti-β tubulin antibody, a housekeeping protein, was performed and the same amount of protein was detected for all samples (Fig. 1, bottom panels).

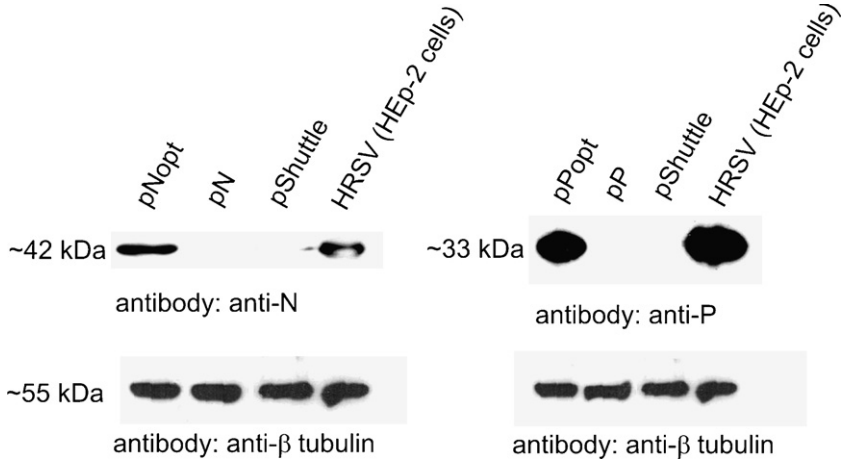

Fig. 1. Western blot analysis of N and P protein expression. Protein extracts were collected from cells transfected with plasmids containing N gene (pN), P gene (pP), optimized N gene (pNopt), optimized P gene (pPopt) or the empty plasmid (pShuttle). HRSV infected cells extracts were also collected and used as positive controls. Polyclonal antibodies against N and P proteins, previously obtained (Simabuco et al., 2007) were used to detect the proteins. A control Western blot was also performed with the samples using an anti-β-tubulin antibody (Santa Cruz). The approximated molecular weight of the proteins is indicated.

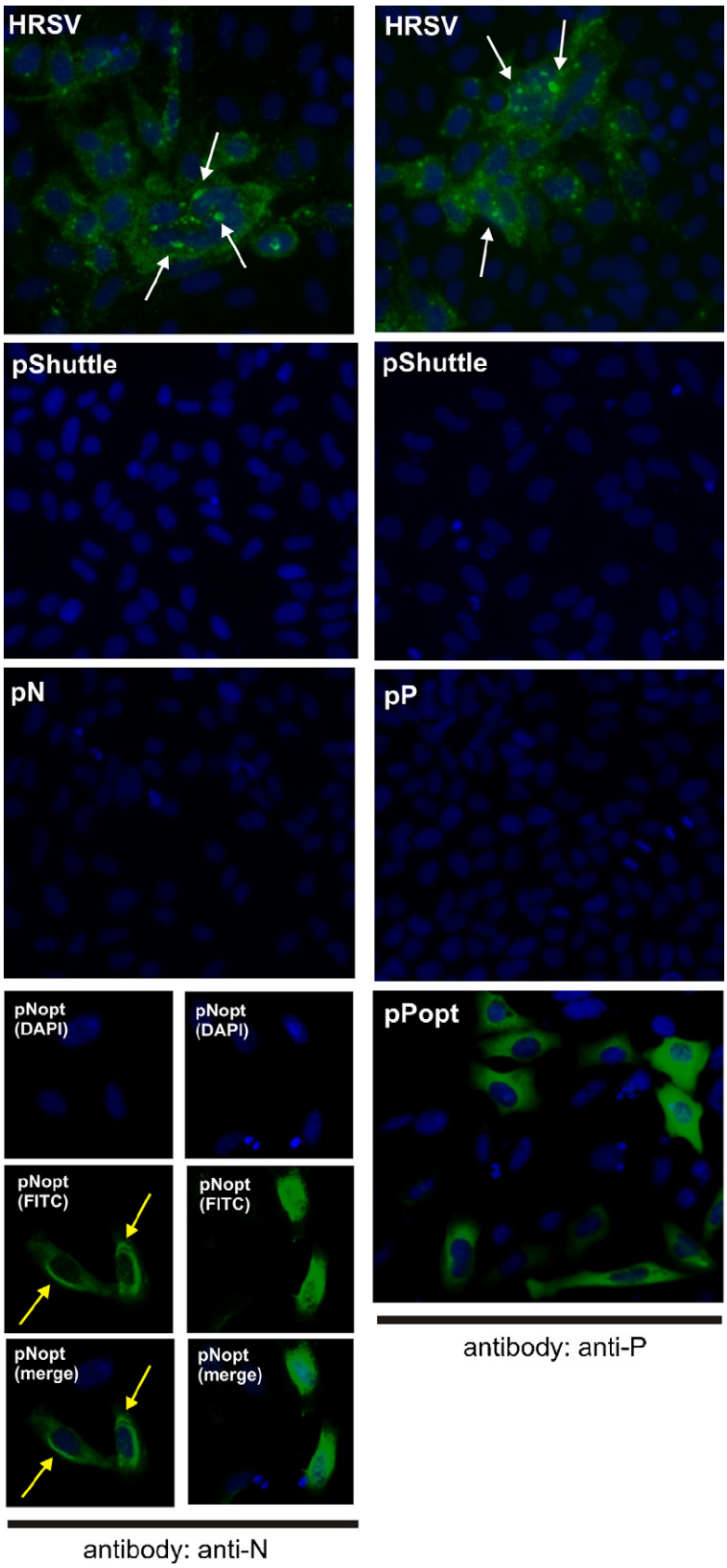

Fig. 2. Immunofluorescence analysis of N and P protein expression. Cells were transfected with plasmids containing N gene (pN), P gene (pP), optimized N gene (pNopt), optimized P gene (pPopt) or the empty plasmid (pShuttle). Cells were also infected with HRSV and used as positive controls. Monoclonal antibodies against N and P proteins previously described (Orvell et al., 1987) were used to detect the proteins. The primary antibodies were detected using an anti-mouse antibody conjugated with FITC. The nucleus staining was made using a DAPI solution (blue). White arrows indicate inclusion bodies and yellow arrows indicate the N protein perinuclear localization. For pNopt the merge and individual staining for nucleus and N protein are shown separately. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

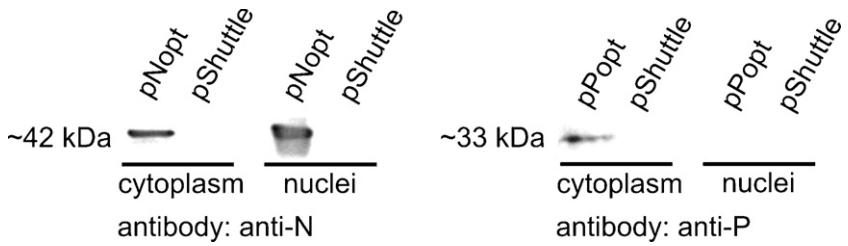

Fig. 3. Western blot analysis of N and P proteins in cytoplasmic and nuclear protein extracts. Cells were transfected with plasmids containing optimized N gene (pNopt), optimized P gene (pPopt) or the empty plasmid (pShuttle). After 72 h, cytoplasmic and nuclear extracts were collected using CelLytic NuCLEAR Extraction Kit (Sigma). Polyclonal antibodies against N and P proteins previously obtained (Simabuco et al., 2007) were used to detect the proteins. The approximated molecular weight of the proteins is indicated.

3.2. Analysis of N and P sub-cellular localization

HEp-2 cells were transfected with pN, pP, pNopt, pPopt or the empty plasmid or they were infected with HRSV. After 48 h, cells were fixed and immunofluorescence was performed. As seen in Fig. 2 (middle panels), no protein expression was detected of the non-optimized genes or in the empty plasmid. Notwithstanding, we detected robust expression of the N and P proteins for pNopt and pPopt, carrying the synthetic genes (bottom panels).

The difference between the cells expressing the recombinant N and P proteins and the HRSV infected cells is remarkable. First, in the infected cells, both N and P proteins show a tendency to concentrate in inclusion bodies (Fig. 2, upper panels, white arrows), which are not present when both proteins are expressed alone (Fig. 2, bottom panels). Thus, the N and P proteins are not able to lead to the formation of inclusion bodies by themselves. Second, for the cells transfected with the optimized genes the P protein is located throughout the entire cytoplasm (Fig. 2, bottom right panel), while the N protein is able to form two different patterns. In one pattern it was located in the cytoplasm and in the nucleus (Fig. 2, pNopt right panels), and in the other it showed a perinuclear localization (Fig. 2, pNopt left panels, yellow arrows).

To confirm the immunofluorescence data, cells were transfected with pNopt, pPopt or pShuttle, after 72 h cytoplasmic and nuclear extracts were collected, and Western blot analyses performed. As seen in Fig. 3, the P protein was detected in the cytoplasm but not in the nucleus, confirming what was observed in the immunofluorescence assays (Fig. 2). The N protein was detected in the cytoplasm and in the nucleus, which is also consistent with the results shown in Fig. 2.

3.3. DNA Immunization with optimized N and P genes

BALB/c mice were immunized with two doses of optimized genes, non-optimized genes, or empty plasmids. After each dose, sera were collected and tested against N and P proteins that had been purified as previously described (Simabuco et al., 2007). As we can see in Fig. 4, for three of five mice immunized with the optimized N gene, high antibody levels were generated against the N protein. On the other hand, for the mice immunized with the non-optimized N gene and for those in the N control cohort, unexpressive antibody levels were generated. Mice immunized with either the optimized or non-optimized P gene, presented low antibody levels; however, the immune response against the optimized P gene was higher than the immune response against the non-optimized gene. All mice of the P control group presented no significant antibody levels (Fig. 4).

In a second immunization experiment, aluminum phosphate was used as adjuvant. As seen in Fig. 5, both Nopt and Popt groups presented robust antibody immune response when compared with

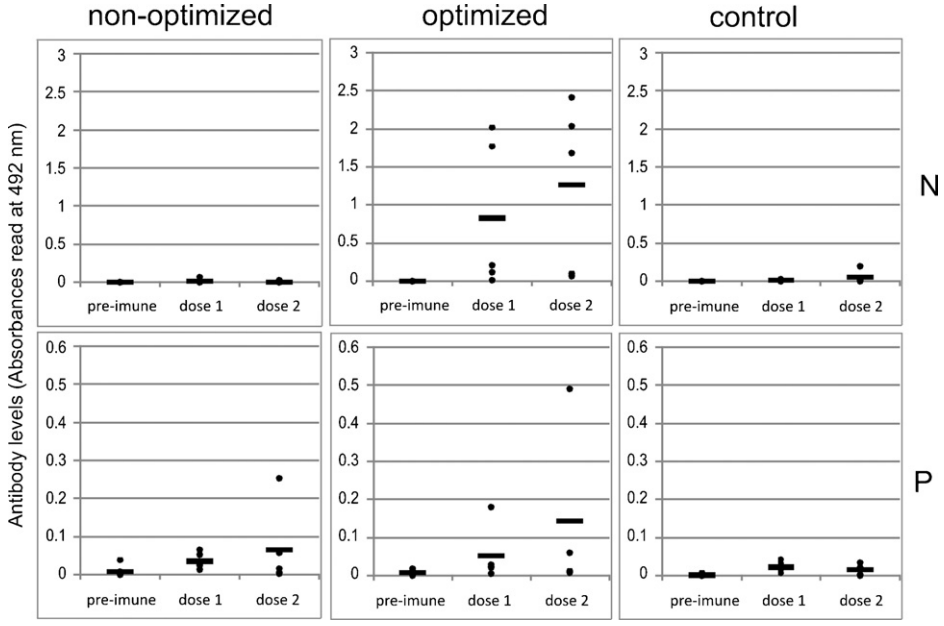

Fig. 4. Antibody levels of mice immunized with optimized N and P genes. Sera dilutions (1:100) were tested against purified N and P proteins previously obtained (Simabuco et al., 2007). Non-optimized N and P genes were also used for comparison and the empty plasmid (pShuttle) was used as a control. The antibody levels were measured by EIA. The averages for each group are presented as black bars.

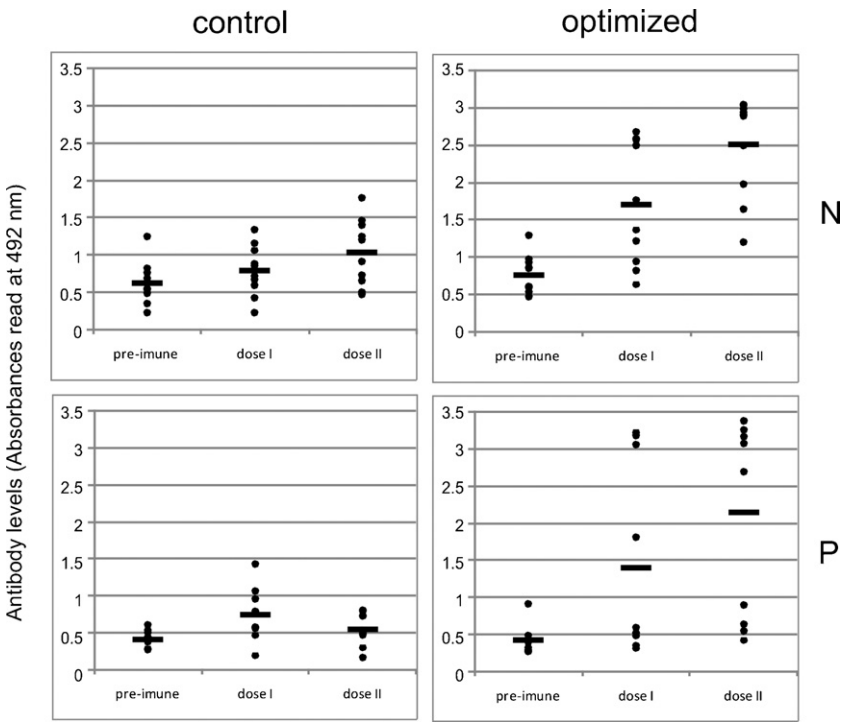

Fig. 5. Antibody levels of mice immunized with optimized N and P genes plus aluminum phosphate adjuvant. Sera dilutions (1:100) were tested against purified N and P proteins previously obtained (Simabuco et al., 2007). The empty plasmid (pShuttle) was used as a control. The antibody levels were measured by EIA. The averages for each group are presented as black bars.

the control after immunization. After the second dose, the antibody levels increased for both Nopt and Popt groups.

4. Discussion

Similar results were found between the Ternette et al. (2007a,b) study of the HRSV F envelope protein and this study of the HRSV N and P nucleocapsid proteins; namely, without optimization of the genes, the expression of HRSV proteins by an RNA polymerase II dependent system is ineffective. Ternette et al. (2007a,b) showed that HRSV genes have several differences when compared with cellular genes. The evolution in the cytoplasmic environment causes the accumulation of numerous modifications in HRSV genes, which would be negatively selected if these genes were transcribed in the cell nucleus. The accumulation of premature polyadenylation sites for example, which hinder the synthesis of full mRNA, was shown to be a critical feature. As shown in this report, the premature poliadenilation sites of the N and P genes were reduced to zero after the optimization process and their CAI values were improved to approximately 0.9.

The results show that no expression is detected when the natural sequences of N and P genes are cloned under the control of an RNA polymerase II promoter (CMVie promoter). However, the same genes when optimized lead to robust expression of the respective proteins when analyzed by either Western blot or immunofluorescence assays (Figs. 1 and 2). The different cellular localizations, observed between the recombinant proteins and those expressed by HRSV, revealed that N and P proteins are unable to aggregate in inclusion bodies by themselves. It is known that the interaction of N, P and L proteins with the viral RNA and cellular structures form the inclusion bodies (García et al., 1993), but more studies are needed to fully understand their individual roles in this process.

Instead of the formation of inclusion bodies, the P protein showed a diffused distribution through the whole cytoplasm, while the N protein was detected in the nucleus or in a perinuclear localization (Fig. 2, bottom panels). The nuclear localization was confirmed by the separation of nuclear and cytoplasmic protein extracts (Fig. 3). It is possible that the N protein may be interacting with cellular RNAs that are presented in the nucleus. The perinuclear localization can be explained by the interaction of the N protein with RNAs that are being exported from the nucleus, or that are being translated at the rough endoplasmic reticulum (Jansen, 2001). It has been shown that the coronavirus N protein contains a nucleolar localization domain and that it can localize to the nucleus and affect cell division (Wurm et al., 2001). The N protein HRSV sequence in the PredictNLS (http://cubic.bioc.columbia.edu/predictNLS/) server was tested, but no nuclear localization sequence was found.

The mice immunization tests revealed that higher antibody levels were generated in mice immunized with N and P optimized genes (Fig. 4). The antibody levels were further increased with aluminum phosphate adjuvant (Fig. 5). It is known that cellular immunity against HRSV avoids the vaccine-enhanced disease observed in the formalin-inactivated vaccine (Becker, 2006; Olson and Varga, 2007) and, since N and P proteins are nucleocapsid proteins, they might have important roles in generating such cellular immunity. It was recently demonstrated that the HRSV N protein has important cellular epitopes (Terrosi et al., 2007). The use of specific peptides, representative of T cell epitopes for N and P proteins, should be considered in further cellular immunity studies using the N and P optimized genes.

In brief, this study shows that the optimization of HRSV N and P genes is essential in generating high levels of expression of the respective proteins. In addition, both proteins presented different intracellular localizations when compared with those expressed by HRSV. The potential of N and P optimized genes as DNA vaccines was also demonstrated in mice immunization tests by the induction of humoral immunity. The data presented indicate that DNA vaccines containing the optimized N and P genes may generate protective immunity against HRSV and to avoid the vaccine-enhanced disease inflammatory effects.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP [Sao Paulo State Research Support Foundation] (proc. # 2003/02041-4) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq [National Council of Scientific and Technological Development] (proc. # 479814/2004-0). F.M.S., R.E.T., C.C. and L.E.F.A. have doctoral degree fellowships from FAPESP. We are grateful to Dr. Erling Norrby, Dr. Ewa Björling and Mariethe Ehnlund (Karolinska Institute, Stockholm, Sweden) for providing the monoclonal antibodies anti-N and anti-P. We also thank Dr. Carlos Menck (São Paulo University, Brazil) for DNA sequencing and fluorescence microscopy support.

References

Becker, Y., 2006. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy. Virus Genes 33, 235–252.

Bowman, M.C., Smallwood, S., Moyer, A., 1999. Dissection of individual functions of the Sendai virus phosphoprotein in transcription. J. Virol. 73, 6474–6483.

Boxus, M., Tignon, M., Roels, S., Toussaint, J.F., Walravens, K., Benoit, M.A., Coppe, P., Letesson, J.J., Letellier, C., Kerkhofs, P., 2007. DNA immunization with plasmids encoding fusion and nucleocapsid proteins of bovine respiratory syncytial virus induces a strong cell-mediated immunity and protects calves against challenge. J. Virol. 81, 6879–6889.

Collins, P.L., Chanock, R.M., Murphy, B.R., 2001. Respiratory syncytial virus. In: Fields, B.N., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E., Knipe, D.M. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1443–1485.

Curran, J., Marq, J.B., Kolakofsky, D., 1995. An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J. Virol. 69, 849–855.

Fulginiti, V.A., Eller, J.J., Sieber, O.F., Joyner, J.W., Minamitani, M., Meiklejohn, G., 1969. Respiratory virus immunization I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 89, 435–438.

García, J., García-Barreno, B., Vivo, A., Melero, J.A., 1993. Cytoplasmic inclusions of respiratory syncytial virus-infected cells: formation of inclusion bodies in transfected cells that co-express the nucleoprotein, the phosphoprotein, and the 22K protein. Virology 195, 243–247.

Hall, C.B., Powell, K.R., Macdonald, N.E., Gala, C.L., Menegus, M.E., Suffin, S.C., Cohen, H.J., 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315, 77–81.

Han, L.L., Alexander, J.P., Anderson, L.J., 1999. Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J. Infect. Dis. 179, 25–30.

Holberg, C.J., Wright, A.L., Martinez, F.D., Ray, C.G., Taussig, L.M., Lebowitz, M.D., 1991. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. Am. J. Epidemiol. 133, 1135–1151.

Horikami, S.M., Curran, J.D., Kolakofsky, D., Moyer, S.A., 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J. Virol. 66, 4901–4908.

Jansen, R.P., 2001. mRNA localization: message on the move. Nat. Rev. Mol. Cell. Biol. 2, 247–256.

Joffe, S., Ray, G.T., Escobar, G.J., Black, S.B., Lieu, T.A., 1999. Cost-effectiveness of respiratory syncytial virus prophylaxis among preterm infants. Pediatrics 104, 419–427.

Kim, H.W., Canchola, J.G., Brandt, C.D., Pyles, G., Chanock, R.M., Jensen, K., Parrot, R.H., 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89, 422–434.

Lemanske, R.F., 2004. Viral infections and asthma inception. J. Allergy Clin. Immunol. 114, 1023–1026.

Meric, C., Spehner, D., Mazarin, V., 1994. Respiratory syncytial virus nucleocapsid protein (N) expressed in insect cells forms nucleocapsid-like structures. Virus Res. 31, 187–201.

Olson, M.R., Varga, S.M., 2007. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. J. Immunol. 179, 5415–5424.

Orvell, C., Norrby, E., Mufson, M.A., 1987. Preparation and characterization of monoclonal antibodies directed against five structural components of human respiratory syncytial virus subgroup B. J. Gen. Virol. 68,3125–3135.

Sasaki, S., Takeshita, F., Xin, K., Ishii, N., Okuda, K., 2003. Adjuvant formulations and delivery systems for DNA vaccines. Methods 31, 243–254.

Schmidt, A.C., Johnson, T.R., Openshaw, P.J.M., Braciale, T.J., Falsey, A.R., Anderson, L.J., Wertz, G.W., Groothuis, J.R., Prince, G.A., Melero, J.A., Graham, B.S., 2004. Respiratory syncytial virus and other pneumoviruses: a review of the international symposium—RSV 2003. Virus Res. 106, 1–13.

Simabuco, F.M., Carromeu, C., Farinha-Arcieri, L.E., Tamura, R.E., Ventura, A.M., 2007. Production of polyclonal antibodies against the human respiratory syncytial virus nucleoprotein and phosphoprotein expressed in *Escherichia coli*. Protein Express. Purif. 53, 209–215.

Spehner, D., Drillien, R., Howley, P.M., 1997. The assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by the phosphoprotein. Virology 232, 260–268.

Taylor, G., Bruce, C., Barbet, A.F., Wyld, S.G., Thomas, L.H., 2005. DNA vaccination against respiratory syncytial virus in young calves. Vaccine 23,1242–1250.

Ternette, N., Stefanou, D., Kuate, S., Überla, K., Grunwald, T., 2007a. Expression of RNA virus proteins by RNA polymerase II dependent expression plasmids is hindered at multiple steps. Virol. J. 4, 51.

Ternette, N., Tippler, B., Uberla, K., Grunwald, T., 2007b. Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. Vaccine 25, 7271–7279.

Terrosi, C., Di Genova, G., Savellini, G.G., Correale, P., Blardi, P., Cusi, M.G., 2007. Immunological characterization of respiratory syncytial virus N protein epitopes recognized by human cytotoxic T lymphocytes. Viral Immunol. 20,399–406.

Trépanier, P., Payment, P., Trudel, M., 1981. Concentration of human respiratory syncytial virus using ammonium sulfate, polyethylene glycol or hollow fiber ultracentrifugation. J. Virol. Methods 3, 201–211.

Ulmer, J.B., DeWitt, C.M., Chastain, M., Friedman, A., Donnelly, J.J., McClements, W.L., Caulfield, M.J., Bohannon, K.E., Volkin, D.B., Evans, R.K., 1999. Enhancement of DNA vaccine potency using conventional aluminum adjuvants. Vaccine 18, 18–28.

Vaughan, K., Rhodes, G.H., Gershwin, L.J., 2005. DNA immunization against respiratory syncytial virus (RSV) in infant rhesus monkeys. Vaccine 23, 2928–2942.

Wurm, T., Chen, H., Hodgson, T., Britton, P., Brooks, G., Hiscox, J.A., 2001. Localization to the nucleolus is a common feature of coronavirus nucleoproteins, and the protein may disrupt host cell division. J. Virol. 75, 9345–9356.