Available online at www.sciencedirect.com
ScienceDirect
Virus Research 133 (2008) 178–186
Virus Research
www.elsevier.com/locate/virusres

Synthesis of recombinant human parainfluenza virus 1 and 3 nucleocapsid proteins in yeast *Saccharomyces cerevisiae*

Mindaugas Juozapaitis [a,*], Aurelija Zvirbliene [a], Indre Kucinskaite [a], Indre Sezaite [a], Rimantas Slibinskas [a], Mayte Coiras [b], Fernando de Ory Manchon [b], María Rosa López-Huertas [b], Pilar Pérez-Breña [b], Juozas Staniulis [c], Irena Narkeviciute [d], Kestutis Sasnauskas [a]

[a] *Institute of Biotechnology, V.A. Graiciuno 8, Vilnius LT-02241, Lithuania*
[b] *National Center of Microbiology, ISCIII, Majadahonda, Madrid, Spain*
[c] *Institute of Botany, Zaliuju ezeru 49, LT-08406 Vilnius, Lithuania*
[d] *Center of Pediatrics, Clinic of Children's Diseases, Vilnius University, Santariskiu 4, LT-08406 Vilnius, Lithuania*

Received 17 September 2007; received in revised form 19 December 2007; accepted 20 December 2007
Available online 4 February 2008

Abstract

Human parainfluenza virus types 1 and 3 (HPIV1 and HPIV3, respectively), members of the virus family *Paramyxoviridae*, are common causes of lower respiratory tract infections in infants, young children, the immunocompromised, the chronically ill, and the elderly. In order to synthesize recombinant HPIV1 and HPIV3 nucleocapsid proteins, the coding sequences were cloned into the yeast *Saccharomyces cerevisiae* expression vector pFGG3 under control of *GAL7* promoter. A high level of recombinant virus nucleocapsid proteins expression (20–24 mg l^{-1} of yeast culture) was obtained. Electron microscopy demonstrated the assembly of typical herring-bone structures of purified recombinant nucleocapsid proteins, characteristic for other paramyxoviruses. These structures contained host RNA, which was resistant to RNase treatment. The nucleocapsid proteins were stable in yeast and were easily purified by caesium chloride gradient ultracentrifugation. Therefore, this system proved to be simple, efficient and cost-effective, suitable for high-level production of parainfluenza virus nucleocapsids as nucleocapsid-like particles. When used as coating antigens in an indirect ELISA, the recombinant N proteins reacted with sera of patients infected with HPIV1 or 3. Serological assays to detect HPIV-specific antibodies could be designed on this basis.

Keywords: Human parainfluenza viruses (HPIV1, HPIV3); Nucleocapsid; Expression; Yeast; Nucleocapsid-like particles (NLPs); ELISA

1. Introduction

Human parainfluenza viruses (HPIV) discovered in the late 1950s belong to *Paramyxoviridae* family, which is a large, growing group of viruses that cause significant human and veterinary disease (for review Chanock et al., 2001; Henrickson, 2003). HPIV are enveloped, and their genomes are organized on a single negative-sense strand of RNA approximately 15,000 nucleotides. These are organized to encode six proteins common to all members within HPIV group (N, P, M, F, HN and L). Various members also encode one or more additional proteins (C, D, V) that are not essential for replication *in vitro* (Karron and Collins, 2007). Inside the viral envelope there is a linear ribonucleoprotein core consisting of a single-stranded genomic RNA molecule to which nucleocapsid proteins (N) are tightly bound, each N protein molecule binding to six nucleotides (Lamb and Kolakofsky, 2001; Chanock et al., 2001). The morphology of N-bound RNA (ribonucleoprotein) is considered one of the defining features of the family *Paramyxoviridae* and is described as a relatively rigid coiled rod ~18–20 nm in diameter which has a herring-bone appearance when imaged under electron microscope. The ribonucleoprotein also contains smaller number of the phosphoprotein (P) and the large (L) polymerase protein, both of which are required to transcribe genomic RNA into mRNA and replicate viral genome. The ribonucleoprotein renders the HPIV genome nuclease resistant (Bhella et al., 2002). There are four genetically and antigenically different types, HPIV types 1–4 (HPIV1–4). HPIV1 and HPIV3 belong to the

* Corresponding author. Tel.: +370 5 2602104; fax: +370 5 2602116.
E-mail address: jmind@ibt.lt (M. Juozapaitis).

0168-1702/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2007.12.016

genus *Respirovirus*, whereas HPIV2 and HPIV4 to the genus *Rubulavirus* (Henrickson and Savatski, 1992, 1996).

HPIV replicate in the epithelial cells that line the respiratory tract, causing the airway illness in humans. HPIV are associated with a rate of community acquired respiratory illness similar to that of the human metapneumovirus and influenza virus (Mullins et al., 2004). The most susceptible groups are infants, young children, the immunocompromised, the chronically ill and the elderly (Glezen et al., 2000; Chanock et al., 2001). Adults infected with these viruses are often affected to a lesser extent. Infections during the adulthood are usually confined to the upper respiratory tract, causing mild symptoms or are asymptomatic (Lamy et al., 1973; Whimbey et al., 1997). In particular, HPIV1 and HPIV3 have been identified as important causes of outbreaks of respiratory tract infections, especially in institutional settings. HPIV causes 30–40% of all acute respiratory infections in infants and children (Murphy et al., 1980; Glezen et al., 1984; Fiore et al., 1998; Henrickson, 2003). These include common cold with fever, croup, bronchiolitis, and pneumonia. HPIV1 is commonly associated with croup. HPIV3 are second only to respiratory syncytial virus as a cause of pneumonia and bronchiolitis in infants and young children. Reinfection with HPIV can occur throughout life, with elderly and immunocompromised persons being at a greater risk of serious complications of infections.

The diagnosis of parainfluenza viruses infection usually requires the detection of virus or viral RNA in clinical specimens, although serological assays using viral antigen play a role and are important for epidemiological studies. The difficulty of *in vitro* culture of these viruses however limits such work to specialized laboratories. After natural infection with HPIV, most children and adults develop measurable levels of IgM, IgG and IgA antibodies in the serum. These antibodies have been shown to be correlated with disease prevention and amelioration in adults. Significant rise in specific IgG antibodies between appropriately collected paired serum specimens or specific IgM antibodies in a single serum specimen could confirm the infection with HPIV (Glezen et al., 1984; Denny and Clyde, 1986; Falsey, 1991; Marx et al., 1997, 1999).

Enzyme-linked immunosorbent assay (ELISA) is by far the most sensitive and easily performed method which has been used to detect HPIV but is the least specific and detects many dual HPIV antibody titers. This problem is greatest when trying to separate HPIV1 infection from HPIV3 infection (Chanock et al., 1958; Henrickson, 2003).

Virus recombinant proteins are promising tools for development of vaccines and diagnostics. Advances in gene expression technology have made the production of recombinant viral proteins in both eukaryotic and prokaryotic host systems easier and more efficient. The ease with which genetic modification can be carried out; the yield of recombinant proteins and the host's capability for post-translation modification often governs the choice of host systems. Yeast are unicellular eukaryotic microorganisms that are capable of performing eukaryotic processing steps on the polypeptides expressed and are easy to manipulate genetically. Since yeast are eukaryotes, their intracellular environment is more suitable for the correct folding of eukaryotic virus proteins (Romanos et al., 1992).

A considerable body of data on formation of homologous and chimeric virus-like and nucleocapsid-like particles (NLPs) of various viruses in yeast has been reported to date (Hofmann et al., 1996; Sasnauskas et al., 1999; Gedvilaite et al., 2000; Hale et al., 2002; Samuel et al., 2002; Juozapaitis et al., 2007a; Freivalds et al., 2006; Kucinskaite et al., 2007).

The expression of recombinant nucleoproteins with spontaneous assembly into nucleocapsid-like particles has been demonstrated for a number of paramyxoviruses using both prokaryotic and eukaryotic systems (Spehner et al., 1991; Hummel et al., 1992; Fooks et al., 1993; Meric et al., 1994; Warnes et al., 1995; Bhella et al., 2002). Recently, the crystal structure of N protein-RNA complex of rabies and vesicular stomatitis viruses has been determined using recombinant N proteins expressed in insect and bacterial cells, respectively (Albertini et al., 2006; Green et al., 2006). It proves that N proteins produced in heterologous protein expression systems might provide researchers with valuable and low-cost material for complex studies of viral protein structure and functions. In addition, we have previously shown that N proteins of various paramyxoviruses self-assemble very efficiently into NLPs in yeast (Slibinskas et al., 2004; Juozapaitis et al., 2005, 2007b). It was also demonstrated that yeast derived recombinant nucleocapsid-like particles of mumps and measles viruses share similar antigenic structure with native viral nucleocapsids and are excellent tools for serodiagnostic of these viruses in sera and oral fluid (Samuel et al., 2002, 2003; Zvirbliene et al., 2007).

Consequently, our aim was to construct efficient high-level yeast expression systems for generation of HPIV1 and HPIV3 N proteins as NLPs with respect to produce large quantities of recombinant HPIV1/3 nucleoproteins for serological assays. The nucleocapsid proteins of paramyxoviruses usually elicit a strong and long-lasting humoral immune response in patients. Therefore, NLPs are excellent tools for detection of virus-specific antibodies in human sera (Ulrich et al., 1998; Pumpens and Grens, 2002; Samuel et al., 2002, 2003). HPIV1 and HPIV3 N proteins share low sequence homology in their C-terminal domain and might be used to develop virus-specific serological assays. Hence, in this study we describe the cloning of HPIV1/3 N genes, production and characterization of recombinant NLPs expressed in yeast *Saccharomyces cerevisiae* and present preliminary data on its use in detecting HPIV1/3-specific IgG in human serum samples by an indirect ELISA.

2. Material and methods

2.1. Strains, media, yeast transformation and cultivation

Bacterial recombinants were screened in *Escherichia coli* DH5αF′ cells. Yeast *S. cerevisiae* AH22 *MATa leu2 his4* was used for expression experiments. Transformation of yeast *S. cerevisiae* cells was performed by conventional methods (Sambrook and Russell, 2001). Selection of transformants resistant to formaldehyde was carried out on the YEPD (yeast extract

1%, peptone 2%, dextrose 2%) agar supplemented with 4 mM formaldehyde as described earlier (Sasnauskas et al., 1992). *S. cerevisiae* transformants were grown in YEPD medium supplemented with 5 mM formaldehyde or in YEPG induction medium (yeast extract 1%, peptone 2%, galactose 3%).

2.2. Cloning of N genes of HPIV1 and HPIV3 into yeast vectors

Prototype strains of HPIV1 (C-35) and HPIV3 (C-243) were used to obtain the nucleoprotein genes. Nucleic acids were subjected to a single step combined RT-PCR amplification reaction using the Promega Access RT-PCR system kit (Promega, San Luis Obispo, USA), which consisted in a PCR mixture containing 2 mM $MgSO_4$, 200 μM each of dATP, dGTP, dCTP, and dTTP, 10 pmol of specific primers for each HPIV, 10 μl of 5× reaction buffer, 5 U of AMV reverse transcriptase (RT), and 5 U of *Tfl* DNA polymerase. Amplifications were carried out in a PTC-200 (Peltier Thermal Cycler, MJ Research, Watertown, USA). An initial cycle of 48 °C for 45 min and 94 °C for 3 min was applied. Cycling conditions of the PCR were 45 cycles: 94 °C for 30 s; 50 °C for 1.5 min; 72 °C for 1 min; a final incubation of 72 °C for 10 min.

RT-PCR primers:

- HPIV1 – primer forward (5′ → 3′) GGACAAGTCACAGACATTTGATCTT; primer reverse (5′ → 3′) CTTTTGATCTGGCTTCTATTGYCCAA.
- HPIV3 – primer forward (5′ → 3′) GGAACTCTATAATTTCAAAAATG; primer reverse (5′ → 3′) TTCGATTAGTTGCTTCCAAATGCATTA.

For cloning into the yeast expression vector, HPIV1 and HPIV3 N genes were reamplified using specific primers, which included a BcuI site for subcloning into the yeast vector, a single ATG codon in the forward primer and a stop TAA codon in the reverse primer. The primer sequences containing the BcuI sites (in bold) and the start and stop codons (underlined) are shown below. For HPIV1:

- Primer forward (5′ → 3′) AA**ACTAGT**ACAATGGCAGGTCTACTAAGTACT; primer reverse (5′ → 3′) AGT**ACTAGT**TTAAATTCCTCCTATCCCTGCC.

For HPIV3:

- Primer forward (5′ → 3′) AGA**ACTAGT**ACAATGTTGAGCCTATTTGATACATTTA; primer reverse (5′ → 3′) GAT**ACTAGT**TTAGTTGCTTCCAAATGCATTAAACAGA).

After amplification, DNA bands corresponding to the HPIV1 N and HPIV3 N genes were excised, gel-purified and cloned into pBluescript II KS (pBSIIKS) plasmid for sequencing. The HPIV1 N and the HPIV3 N gene sequences isolated from pBSIIKS were ligated into the BcuI site of the yeast expression vector pFGG3 under control of *GAL7* promoter (Slibinskas et al., 2004). The resulting plasmids pFGG3-HPIV1-N and pFGG3-HPIV3-N, each separately, were used for transformation of yeast *S. cerevisiae* strain AH22 (*leu2 his 4*).

All DNA manipulations were performed according to the standard procedures (Sambrook and Russell, 2001). Enzymes and kits for DNA manipulations were purchased from Fermentas UAB (Vilnius, Lithuania).

2.3. Expression and purification of nucleocapsid-like particles from yeast

The procedure used for expression of N genes in *S. cerevisiae* cells was similar to that described earlier (Samuel et al., 2002). Briefly, *S. cerevisiae* cells harbouring pFGG3-HPIV1-N and pFGG3-HPIV3-N plasmids were inoculated into YEPD media supplemented with 5 mM formaldehyde, grown overnight and reinoculated into YEPG induction media and cultured for 18 h at 28 °C. The cells were harvested by centrifugation and stored at −70 °C. For the purification, 5 g of yeast biomass harbouring pFGG3-HPIV1-N and pFGG3-HPIV3-N, separately, resuspended in PBS containing 10 mM EDTA to inhibit metalloproteinases and 1 mM PMSF, were disrupted by homogenization in the presence of glass beads. The yeast-expressed N proteins were purified by successive ultracentrifugation through 30% sucrose cushion (100,000 × *g*, 3 h) followed by two ultracentrifugations in CsCl gradient ranging from 1.23 to 1.38 g ml^{-1} (48 h, 100,000 × *g*). Fractions containing proteins were identified by SDS-PAGE. After first centrifugation in CsCl gradient fractions containing proteins with the molecular weight corresponding to native HPIV1 and HPIV3 N proteins (60 and 64 kDa, respectively) were pooled, diluted with 1.31 g ml^{-1} CsCl and repeatedly centrifuged in CsCl gradient. Fractions were collected and those containing recombinant N protein were pooled and dialyzed against PBS for electron microscopy (EM) (Sasnauskas et al., 1999; Samuel et al., 2002). The buoyant density of the fractions was determined with a refractometer.

2.4. Electron microscopy

After purification by CsCl centrifugation suspensions of yeast-expressed N proteins were placed on 400-mesh carbon coated palladium grids. The samples were stained with 2% aqueous uranyl acetate solution and examined with JEM-100S electron microscope.

2.5. SDS-PAGE and Western blotting

Samples were boiled in a reducing sample buffer and gel electrophoresis was run in SDS-Tris-glycine buffer. Proteins were stained by the addition of Coomassie brilliant blue (Sigma–Aldrich Co., St. Louis, USA). After SDS-PAGE electrophoresis proteins were transferred to Immobilon P membrane (Millipore, Bedford, MA, USA) as described in Sambrook and Russell (2001). The blots were blocked with 5% milk in PBS for 2 h. The blocking solution was removed and the blots were incubated with rabbit (for HPIV1) or goat (for HPIV3) polyclonal sera diluted 1:1000 or HPIV-IgG positive human serum

specimens diluted 1:200. The corresponding horseradish peroxidase (HRP)-labeled anti-rabbit, anti-goat IgG or anti-human IgG conjugates (Dako, Glostrup, Denmark) for detection of specific antibody binding were used (1:1000). The blots were stained with the TMB substrate (Fermentas UAB, Vilnius, Lithuania).

2.6. Stability of viral particles

The particles were very stable in CsCl solution at 4 °C. Dialyzed samples were stored at −20 °C in PBS with 40% glycerol. The stability of particles was tested by incubating with 20 mM EDTA, 10 mm EGTA or 5 mM DTT, 1 h, at 37 °C. After incubation electron microscopy analysis was carried out.

2.7. Native agarose gel electrophoresis and ribonuclease treatment

To prove the association of purified N proteins with nucleic acid of cellular origin, 20 μg of each protein was separated in a native 1.2% agarose gel electrophoresis. Thereafter, the gel was stained by ethidium bromide, photographed under an UV transilluminator and subsequently stained by Coomassie brilliant blue.

A total of 20 μg of each caesium chloride gradient-purified recombinant protein in 20 μl PBS solution were treated with 1 μl of ribonuclease (RNase) A/T1 mix, containing 10 units of RNase A and 5 units of RNase T1 (Fermentas, Vilnius, Lithuania) at 37 °C for 30 min. After incubation, 20 μg of each recombinant N protein, either RNase-treated samples or samples incubated without RNase, were analyzed by native agarose gel electrophoresis.

2.8. ELISA test

96-Well microtiter plates (Nerbe-Plus, Germany) were coated with recombinant HPIV1 or HPIV3 recombinant N protein at a concentration of 5 μg ml^{-1} in 0.05 M Na-carbonate buffer (pH 9.5) by incubation overnight at 4 °C. The plates were blocked with 2% bovine serum albumin in PBS and then incubated for 1 h at 37 °C with serum samples diluted 1:100 in PBS containing 0.1% Tween-20. After washing, the plates were incubated with peroxidase-labeled secondary antibody against human IgG (Sigma) and developed using TMB substrate (Sigma). Optical density (OD) was measured in a microplate reader (Tecan, Groedig, Austria) at a wavelength 450 nm.

2.9. Serum specimens

A total of 16 samples from 11 cases of HPIV infection in children, as diagnosed by direct detection on respiratory sample, were studied. Diagnosis of HPIV was performed directly in the cells of the respiratory secretions (nasopharyngeal aspirates) of children presenting acute respiratory illness by indirect immunofluorescence (IF) staining using monoclonal antibodies (Chemicon International, Temecula, CA), and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma). Seven samples were taken in the acute phase of the disease, and 9 in the convalescent one, 15–21 days after the onset of disease. Thus, in five cases paired serum samples were available (Table 1). Serum samples were tested for a presence of IgG antibodies against HPIV1/2/3 using commercial ELISA kit (IBL, Hamburg, Germany). In the commercial kit, the plates are coated with HPIV1, HPIV2, HPIV3 (virus strains ATCC VR-92, VR-93, VR-105, respectively) disrupted by ultrasonication (manufacturer's information).

Table 1
Immunoreactivity of recombinant yeast-derived HPIV1 and HPIV3 NLPs with serum antibodies of patients with confirmed HPIV infection

Case[a]	IBL hPIV1/2/3 IgG ELISA kit		rHPIV1 N IgG ELISA		rHPIV3 N IgG ELISA		HPIV type	Serological classification
1-a	0.32	+/−	3.39	+++	3.32	+++	HPIV1	Cross-reactivity
1-c	2.16	+++	3.29	+++	3.19	+++		
2-a	0.10	−	0.39	+/−	0.43	+/−		
2-c	0.46	+/−	2.97	+++	0.55	+		HPIV1
3-a	0.09	−	0.22	−	0.29	−		
3-c	0.33	+/−	0.23	−	2.35	+++		HPIV3
4-a	0.31	+/−	0.82	+	2.73	+++		
4-c	0.42	+/−	1.21	++	2.45	+++		Cross-reactivity
5-a	0.14	−	0.07	−	0.10	−		Negative
5-c	0.13	−	0.07	−	0.10	−		
6-a	0.58	+	0.35	+/−	0.79	+	HPIV3	HPIV3
7-a	0.36	+/−	0.29	−	0.98	+		HPIV3
8-c	0.16	−	2.56	+++	0.44	+/−	HPIV1	HPIV1
9-c	0.09	−	0.29	−	0.47	+/−		Probable HPIV3
10-c	0.10	−	0.39	+/−	0.48	+/−		Cross-reactivity
11-c	0.12	−	0.25	−	0.49	+/−		Probable HPIV3
Control serum samples from IBL hPIV1/2/3 IgG ELISA kit								
Positive	2.39	+++	2.93	+++	3.06	+++		
Negative	0.06	−	0.08	−	0.08	−		

+++: OD value, >1.5; ++: OD value, 1.0–1.5; +: OD value, 0.5–1.0; +/−: OD value, 0.3–0.5; −: OD value, <0.3.
[a] a: acute sample; c: convalescent sample.

Serum samples were assayed and found negatives for a panel of respiratory agents, including influenza A and B, adenovirus, respiratory syncytial virus, *Mycoplasma pneumoniae*, *Coxiella burnetti* and *Chlamydia pneumoniae*, by complement fixation test.

3. Results and discussion

3.1. High-level expression of N proteins of HPIV1 and HPIV3 in yeast

Nucleocapsid proteins of the *Paramyxoviridae* family share some structural homology between different members of the family (Miyahara et al., 1992; Bhella et al., 2002). HPIV1 and HPIV3 nucleocapsid proteins share 58% identity. The main amino acid sequence differences are localized at the C-terminal end of proteins (Fig. 1).

Expression of recombinant N proteins was analyzed in SDS-PAGE. SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring pFGG3-HPIV1-N and pFGG3-HPIV3-N, after induction with galactose revealed the presence of an additional protein bands of approximately 60 and 64 kDa, respectively (Fig. 2A and B, lanes 2), which is consistent with the molecular weight of N proteins of HPIV reported in literature (Miyahara et al., 1992; Henrickson, 2003). No additional bands of the corresponding molecular size were observed in crude lysates of *S. cerevisiae*, harbouring vector pFGG3 (Fig. 2A and B, lanes 1). SDS-PAGE analysis revealed that both N proteins were expressed at high level.

3.2. Purification of recombinant proteins

After centrifugation through 30% sucrose cushion recombinant N proteins of both viruses were found in pellets (data not shown), which is consistent with their large size and multimeric organization. Furthermore, ultracentrifugation of the resolubilized pellets in CsCl gradient and analysis revealed recombinant N proteins in fractions with buoyant densities of 1.28–1.30 g ml^{-1}. This buoyant density is characteristic of most virus-like particles or nucleocapsid-like structures with or without nucleic acids (Thorne and Dermott, 1976; Buchholz et al., 1993; Candrika et al., 1995; Sasnauskas et al., 1999). These fractions contain highly purified N proteins with only minor

```
                1                                                  50
hPIV1 N   (1)  MAGLLSTFDTFSSRRSESINKSGGGAIIPGQRSTVSVFILGPSVTDDADK
hPIV3 N   (1)  ---MLSLFDTFNARRQENITKSAGGAIIPGQKNTVSIFALGPTITDDNEK
               51                                                 100
hPIV1 N  (51)  LLIATTFLAHSLDTDKQHSQRGGFLVSLLAMAYSSPELYLTTNGVNADVK
hPIV3 N  (48)  MTLALLFLSHSLDNEKQHAQRAGFLVSLLSMAYANPELYLTTNGSNADVK
               101                                                150
hPIV1 N (101)  YVIYNIERDPKRTKTDGFIVKTRDMEYERTTEWLFGPMIN--KNPLFQGQ
hPIV3 N  (98)  YVIYMIEKDLKRQKYGGFVVKTREMIYEKTTDWIFGSDLDYDQETMLQNG
               151                                                200
hPIV1 N (149)  RENADLEALLQTYGYPACLGAIIVQVWIVLVKAITSSAGLRKGFFNRLEA
hPIV3 N (148)  RNNSTIEDLVHTFGYPSCLGALIIQIWIVLVKAITSISGLRKGFFTRLEA
               201                                                250
hPIV1 N (199)  FRQDGTVKSALVFTGDTVEGIGAVMRSQQSLVSLMVETLVTMNTSRSDLT
hPIV3 N (198)  FRQDGTVQAGLVLSGDTVDQIGSIMRSQQSLVTLMVETLITMNTSRNDLT
               251                                                300
hPIV1 N (249)  TLEKNIQIVGNYIRESGLASFMNTIKYGVETKMAALTLSNLRPDINKLRS
hPIV3 N (248)  TIEKNIQIVGNYIRDAGLASFFNTIRYGIETRMAALTLSTLRPDINRLKA
               301                                                350
hPIV1 N (299)  LVDIYLSKGARAPFICILRDPVHGEFAPGNYPALWSYAMGVAVVQNKAMQ
hPIV3 N (298)  LMELYLSKGPRAPFICILRDPIHGEFAPGNYPAIWSYAMGVAVVQNRAMQ
               351                                                400
hPIV1 N (349)  QYVTGRTYLDMEMFLLGQAVAKDADSKISSALEEELGVTDTAKERLRHHL
hPIV3 N (348)  QYVTGRSYLDIDMFQLGQAVARDAEAQMSSTLEDELGVTHEAKESLKRHI
               401                                                450
hPIV1 N (399)  TNLSGGDGAYHKPTGGGAIEVAIDHTDITFGAEDTADRDNKNWTNNSNER
hPIV3 N (398)  RNINSSETSFHKPTGGSAIEMAIGEEPEQFEHRADQEQDGEPQSSIIQYA
               451                                                500
hPIV1 N (449)  WMN-HSINNHTITISGAEELEEETNDEDITDIENKIARRLADRKQRLSQA
hPIV3 N (448)  WAEGNRSDDRTEQATESDNIKTEQ----Q-NIRDRLNKRLNDRKKQGSQP
               501                 527
hPIV1 N (498)  NNKQDANSDADYENDDDATAAAGIGGI
hPIV3 N (493)  S--TNPTNRTNQDEIDDLFNAFGSN--
```

Fig. 1. Alignment of amino acid sequences of HPIV1 (GeneBank accession no. EU346886) and HPIV3 (GeneBank accession no. EU346887) N proteins. Identical and similar aa are highlighted in grey and dark grey, respectively.

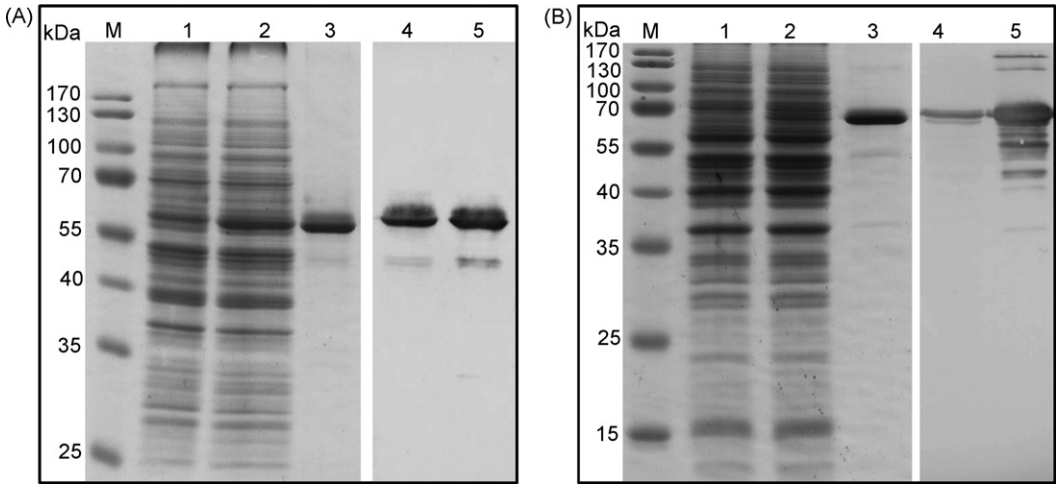

Fig. 2. SDS-PAGE (lanes 1–3) and Western blot (lanes 4 and 5) analysis of yeast lysates and samples of yeast-expressed HPIV1 (A) and HPIV3 (B) N proteins after ultracentrifugation in CsCl. 5 μg of purified N protein (A and B, lanes 3 and 5) or 20 μg of yeast lysates (A and B, lanes 1, 2 and 4) were separated in a 12% SDS-PAGE and stained with Coomassie brilliant blue. After separation proteins HPIV1 (A, lanes 4 and 5) was analyzed by Western blot using hyperimmune rabbit sera generated by immunizing rabbits with purified Sendai virions (Juozapaitis et al., 2005) and HPIV3 (B, lanes 4 and 5) was analyzed using goat anti-parainfluenza virus 2 and 3 polyclonal antibodies (Chemicon International, Cat. No. AB1070). Lanes 1: *Saccharomyces cerevisiae* [pFGG3] lysate; lanes 2 and 4: (A) *S. cerevisiae* [pFGG3-HPIV1-N] lysate, (B) *S. cerevisiae* [pFGG3-HPIV3-N] lysate; lane 3 and 5: CsCl purified HPIV1 (A) and HPIV3 (B) N proteins; M: protein molecular mass marker (Fermentas UAB, Vilnius, Lithuania).

contaminants observed in SDS-PAGE. The SDS-PAGE analysis of purified N proteins after centrifugation in CsCl revealed bands of ~60 and ~64 kDa for HPIV1 and HPIV3, respectively (Fig. 2A and B, lanes 3), which were also observed in crude lysates (Fig. 2A and B, lanes 2). The identity of recombinant HPIV1 N proteins was confirmed by immunoblot with hyperimmune rabbit sera generated by immunizing a rabbit with purified Sendai virus (closely related murine parainfluenza virus type 1 which N protein shares high amino acid sequence homology with HPIV1) virions (Neubert et al., 1991). The identity of HPIV3 N proteins was confirmed with commercial polyclonal goat anti-HPIV3 antibodies (Chemicon International, Cat. No. AB1070).

3.3. Electron microscopy analysis

Electron microscopy of the purified N proteins revealed that both recombinant N proteins of HPIV form nucleocapside-like herring-bone structures and rings which correspond to shorter nucleocapsids viewed along their axis (Fig. 3A and B). The longest rods (the average length is 120 and 200 nm for HPIV1 and HPIV3 yeast-derived NLPs, respectively) were observed after sucrose centrifugation, and each additional step of purification shortened the length of rods. The average lengths of HPIV1 recombinant NLPs were 105 and 90 nm, and for HPIV3 yeast-derived NLPs—190 and 170 nm, respectively after the first and the second CsCl gradient centrifugations. As demonstrated by EM, recombinant HPIV1 N proteins form various nucleocapside-like structures in length and therefore some of them appear as rings (Fig. 3A). In the case of HPIV3, the lengthy rods and the low abundance of ring structures suggest a relatively high intrinsic stability in nucleocapsids formed by heterologically expressed N protein (Fig. 3B). N proteins of HPIV produced in yeast expression system were similar to those previously observed for other paramyxovirus nucleocapsids, expressed in different systems (Rima, 1983; Fooks et al., 1993; Meric et al., 1994; Griffin, 2001; Samuel et al., 2003; Juozapaitis et al., 2005). Formation of NLPs in yeast occurs in the absence of other viral proteins. If host cells proteins are involved in formation of NLPs, they appear to be conserved in both yeast and mammalian cells.

3.4. Yield and stability of recombinant N proteins

In several preparative procedures, the yield of purified HPIV1 and HPIV3 recombinant N proteins was found to be 18–24 mg l^{-1} of induced yeast culture. Recombinant particles were stable in CsCl solution at 4 °C. Treatment with 20 mM EDTA, 10 mM EGTA or 5 mM DTT did not cause dissociation of recombinant particles indicating the assembled structures does not require divalent ions. Dialyzed samples were stored at −20 °C in PBS containing 10 mM EDTA and 40% glycerol.

The yield of recombinant proteins resembles that observed previously for proteins of different other viruses proteins generated in yeast (Sasnauskas et al., 1999; Samuel et al., 2002; Razanskiene et al., 2004). The yeast system employed in the present study proved to be suitable for the efficient expression of N proteins of HPIV. Also, these proteins produced by this system are extremely stable and show no significant degradation when expressed, purified or stored for a long period of time at −20 °C.

3.5. The yeast-expressed N proteins' interaction with nucleic acids

In line with the previous study of yeast-expressed Sendai virus N protein (Juozapaitis et al., 2005), analysis of HPIV1 and 3 N proteins in a native agarose gel electrophoresis revealed their interaction with host RNA, which was resistant to RNase treatment (data not shown).

3.6. Immunoreactivity of yeast-expressed HPIV1 and HPIV3 N proteins with human sera

The immunoreactivity of the recombinant HPIV1 and HPIV3 N proteins was tested in an indirect ELISA format using serum

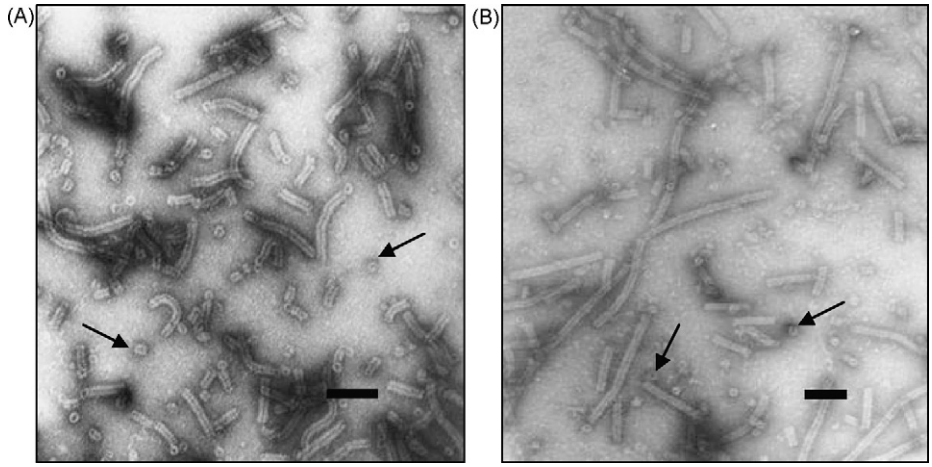

Fig. 3. Electron micrographs of negatively stained HPIV1 (A) and HPIV3 (B) N proteins isolated from *S. cerevisiae*. Scale bar: 100 nm. Arrows indicate ring-like structures.

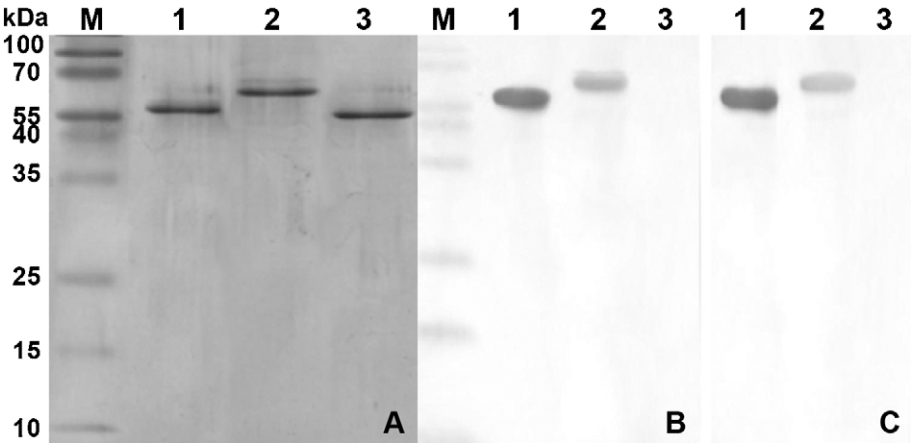

Fig. 4. Immunoreactivity of recombinant HPIV1 and HPIV3 N proteins with serum specimens #1-c (B) and #1-a (C) from a patient with confirmed HPIV1 infection (Table 1). SDS-PAGE (A) and Western blots (B and C) analysis of purified HPIV1 N protein (lanes 1), HPIV3 N protein (lanes 2) and yeast-expressed lyssavirus (CVS) N protein (negative control, lanes 3; Kucinskaite et al., 2007). Recombinant proteins were separated in a 12% SDS-PAGE (A–C) and stained with Coomassie brilliant blue (A). M: protein molecular mass marker (Fermentas UAB, Vilnius, Lithuania).

samples collected from HPIV-infected patients. In five cases paired serum samples were available. In two of them, seroconversion to the homologue type from negative to positive (case 3, due to HPIV3), and from low positive to positive (case 2, due to HPIV1) was detected. Case 1 (with a positive identification of HPIV1) and case 4 showed positive result for both HPIV1 and 3, both in acute and convalescent sample. Case 5 showed no antibodies to HPIV1 and HPIV3. In the cases with single serum sample and typification available, the serological study confirmed the diagnosis (cases 6 and 8). Three cases (7,9 and 11) were classified as probable HPIV3, due to the reactivity to this virus.

The observed cross-reactivity of HPIV1 and HPIV3 N proteins with serum antibodies (as in case 1 with confirmed HPIV1 infection) is explained by a high level of homology between HPIV1 and HPIV3 N protein sequences (Fig. 1). Six serum specimens equivocal (+/−) for HPIV-IgG in commercial ELISA system were found to be positive in NLP-based anti-HPIV1/3 IgG assays. Thus, low levels of anti-HPIV IgG developed at an early stage of the infection were not detectable by a commercial assay but were detectable in NLP-based assays. The discrepancy between NLP-based and commercial test might be explained by the fact that commercial kit is based on the use of total viral proteins with a relatively low content of viral nucleocapsids. It is well documented that most paramyxoviruses induce a strong and long-lasting antibody response directed against nucleocapsid proteins (Ulrich et al., 1998; Pumpens and Grens, 2002; Samuel et al., 2002, 2003).

To confirm the specificity of NLP-based assays, control serum samples (negative and positive for HPIV-IgG, respectively) from the commercial kit were analyzed. The negative control was non-reactive, and the positive control was strongly reactive in NLP-based ELISAs (Table 1). The reactivity of NLPs with serum antibodies suggests that recombinant HPIV1 and HPIV3 N proteins show antigenic similarity with viral nucleocapsids and mimic infectious virus in the presentation of surface-exposed immunodominant epitopes.

To confirm the reactivity of serum antibodies with recombinant HPIV1 and HPIV3 N proteins, Western blot analysis with HPIV-IgG-positive serum samples was performed. Protein bands corresponding to the MW of HPIV1 and HPIV3 N proteins (60 and 64 kDa, respectively) specifically immunostained with serum HPIV-IgG were detected (Fig. 4, lanes 2 and 3).

These data demonstrate that recombinant yeast-derived NLPs are specifically recognized by serum antibodies developed during HPIV1/HPIV3 infection and therefore might be suitable for the development of serological tests for the detection of HPIV1/HPIV3-specific antibodies.

4. Conclusions

Many viral surface and nucleocapsid proteins when expressed in animal host in the absence of other viral gene products have the intrinsic capacity to self-assemble into virus-like particles (for review Pumpens and Grens, 1999, 2002; Ulrich et al., 1998). The active genome of the negative-stranded RNA viruses is found in the form of a nucleocapsid. In the nucleocapsid, the single-stranded RNA is tightly associated with the nucleocapsid proteins in a rod-shaped helical structure. The interaction between RNA and N subunits can be so stable that the nucleocapsid does not disintegrate in high salt concentration, even upon density equilibrium centrifugation (Lamb and Kolakofsky, 2001). For a number viruses within the *Paramyxoviridae* familly, expression of the N protein alone, results in assembly of nucleocapsid-like structures around cellular RNA (Bhella et al., 2002; Juozapaitis et al., 2005). In this study we have demonstrated, that yeast *S. cerevisiae* is an excellent host for a high-level production of HPIV N proteins as NLPs. The immunoreactivity of recombinant yeast-derived NLPs with HPIV-specific IgG antibodies from human sera confirms their antigenic similarity with viral nucleocapsids. Thus, the HPIV NLPs described above represent useful tools for the development of new serological detection systems for HPIV1 and HPIV3. The current study demonstrates the effectiveness of yeast as a host

for generation of recombinant proteins organized in complex structures similar to native viral nucleocapsids.

Acknowledgements

This work was supported by the grant no. B11 from the Lithuanian Science and Study Foundation. Dr. Juan Echevarría (National Center of Microbiology, ISCIII, Madrid, Spain) kindly provided reference strains of the human parainfluenza viruses types 1 and 3.

References

Albertini, A.A.V., Wernimont, A.K., Muziol, T., Ravelli, R.B.G., Clapier, C.R., Schoehn, G., Weissenhorn, W., Ruigrok, R.W.H., 2006. Crystal structure of the rabies virus nucleoprotein-RNA complex. Science 313, 360–363.

Bhella, D., Ralph, A., Murphy, L.B., Yeo, R.P., 2002. Significant differences in nucleocapsid morphology within the *Paramyxoviridae*. J. Gen. Virol. 83, 1831–1839.

Buchholz, C.J., Spehner, D., Drillien, R., Neubert, W.J., Homann, H.E., 1993. The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. J. Virol. 67, 5803–5812.

Candrika, R., Myers, T., Moyer, S.A., 1995. Measles virus nucleocapsid protein can function in Sendai virus defective interfering particle genome synthesis in vitro. Virology 206, 777–782.

Chanock, R.M., Parrott, R.H., Bell, J.A., Rowe, W.P., Huebner, R.J., 1958. New viruses observed in children with respiratory diseases. Public Health Rep. 73, 193–196.

Chanock, R.M., Murphy, B.R., Collins, P.L., 2001. Parainfluenza viruses. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1341–1379.

Denny, F.W., Clyde Jr., W.A., 1986. Acute lower respiratory infections in non-hospitalized children. J. Pediatr. 108, 635–646.

Falsey, A.R., 1991. Noninfluenza respiratory virus infection in long-term care facilities. Infect. Control Hosp. Epidemiol. 12, 602–608.

Fiore, A.E., Iverson, C., Messmer, T., Erdman, D., Lett, S.M., Talkington, D.F., Anderson, L.J., Fields, B., Carlone, G.M., Breiman, R.F., Cetron, M.S., 1998. Outbreak of pneumonia in a long-term care facility: antecedent human parainfluenza virus 1 infection may predispose to bacterial pneumonia. J. Am. Geriatr. Soc. 46, 1112–1117.

Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., Wilkinson, G.W.G., 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J. Gen. Virol. 74, 1439–1444.

Freivalds, J., Dislers, A., Ose, V., Skrastina, D., Cielens, I., Pumpens, P., Sasnauskas, K., Kazaks, A., 2006. Assembly of bacteriophage Qbeta virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. J. Biotechnol. 123, 297–303.

Gedvilaite, A., Frömmel, C., Sasnauskas, K., Burkhard, M., Özel, M., Behrsing, O., Staniulis, J., Jandrig, B., Scherneck, S., Ulrich, R., 2000. Formation of immunogenic virus-like particles by inserting epitopes into surface-exposed regions of hamster polyomavirus major capsid protein. Virology 273, 21–35.

Glezen, W.P., Frank, A.L., Taber, L.H., Kasel, J.A., 1984. Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. J. Infect. Dis. 150, 851–857.

Glezen, W.P., Greenberg, S.B., Atmar, R.L., Piedra, P.A., Cough, R.B., 2000. Impact of respiratory virus infections on persons with chronic underlying conditions. JAMA 283, 499–505.

Green, T.J., Zhang, X., Wertz, G.W., Luo, M., 2006. Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. Science 313, 357–360.

Griffin, D.E., 2001. Measles virus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins Publ., Philadelphia, pp. 1401–1441.

Hale, A.D., Bartkeviciute, D., Dargeviciute, A., Jin, L., Knowles, W., Staniulis, J., Brown, D.W.G., Sasnauskas, K., 2002. Expression and antigenic characterization of the major capsid proteins of human polyomaviruses BK and JC in *Sacharomyces cerevisiae*. J. Virol. Meth. 104, 93–98.

Henrickson, K.J., 2003. Parainfluenza viruses. Clin. Microbiol. Rev. 16, 242–264.

Henrickson, K.J., Savatski, L.L., 1992. Genetic variation and evolution of human parainfluenza virus type 1 hemagglutinin neuraminidase: analysis of 12 clinical isolates. J. Infect. Dis. 166, 995–1005.

Henrickson, K.J., Savatski, L.L., 1996. Two distinct human parainfluenza virus type 1 genotypes detected during the 1991 epidemic. J. Clin. Microbiol. 34, 695–700.

Hofmann, K.J., Neeper, M.P., Marcus, H.Z., Brown, D.R., Muller, M., Jansen, K.U., 1996. Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation HPV-18 virus-like particles in *Saccharomyces cerevisiae*. J. Gen. Virol. 77, 465–468.

Hummel, K.B., Erdman, D.D., Heath, J., Bellini, W.J., 1992. Baculovirus expression of the nucleoprotein gene of measles virus and utility of the recombinant protein in diagnostic enzyme immunoassay. J. Clin. Microbiol. 30, 2874–2880.

Juozapaitis, M., Slibinskas, R., Staniulis, J., Sakaguchi, T., Sasnauskas, K., 2005. Generation of Sendai virus nucleocapsid-like particles in yeast. Virus Res. 108, 221–224.

Juozapaitis, M., Serva, A., Kucinskaite, I., Zvirbliene, A., Slibinskas, R., Staniulis, J., Sasnauskas, K., Shiell, B.J., Bowden, T.R., Michalski, W.P., 2007a. Generation of menangle virus nucleocapsid-like particles in yeast *Saccharomyces cerevisiae*. J. Biotechnol. 130, 441–447.

Juozapaitis, M., Serva, A., Zvirbliene, A., Slibinskas, R., Staniulis, J., Sasnauskas, K., Shiell, B.J., Wang, L.F., Michalski, W.P., 2007b. Generation of henipavirus nucleocapsid proteins in yeast *Saccharomyces cerevisiae*. Virus Res. 124, 95–102.

Karron, R.A., Collins, P.L., 2007. Parainfluenza viruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 5th ed. Lippincott Williams & Wilkins Publ., Philadelphia, pp. 1497–1526.

Kucinskaite, I., Juozapaitis, M., Serva, A., Zvirbliene, A., Johnson, N., Staniulis, J., Foks, A.R., Muller, T., Sasnauskas, K., Ulrich, R., 2007. Antigenic characterization of yeast-expressed lyssavirus nucleoproteins. Virus Genes 35, 521–529.

Lamb, R.A., Kolakofsky, D., 2001. Paramyxoviridea: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins Publ., Philadelphia, pp. 1305–1340.

Lamy, M.E., Pouthier-Simon, F., Debacker-Williame, E., 1973. Respiratory viral infections in hospital patients with chronic bronchitis. Observations during periods of exacerbation and quiescence. Chest 63, 336–341.

Marx, A., Török, T.J., Holman, R.C., Clarke, M.J., Anderson, L.J., 1997. Pediatric hospitalizations for croup (laryngotracheobronchitis): biennial increases associated with human parainfluenza virus 1 epidemics. J. Infect. Dis. 176, 1423–1427.

Marx, A., Gary, H.E., Marston, B.J., Erdman, D.D., Breiman, R.F., Török, T.J., Plouffe, J.F., File, T.M., Anderson, L.J., 1999. Parainfluenza virus infection among adults hospitalized for lower respiratory tract infection. Clin. Infect. Dis. 29, 134–140.

Méric, 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.

Miyahara, K., Kitada, S., Yoshimoto, M., Matsumura, H., Kawano, M., Komada, H., Tsurudome, M., Kusagawa, S., Nishio, M., Ito, Y., 1992. Molecular evolution of human paramyxoviruses. Nucleotide sequence analyses of the human parainfluenza type 1 virus NP and M protein genes and construction of phylogenetic trees for all the human paramyxoviruses. Arch. Virol. 124, 255–268.

Mullins, J.A., Erdman, D.D., Weinberg, G.A., Edwards, K., Hall, C.B., Walker, F.J., Iwane, M., Anderson, L.J., 2004. Human metapneumovirus infection among children hospitalized with acute respiratory illness. Emerg. Infect. Dis. 10, 700–705.

Murphy, B., Phelan, P.D., Jack, I., Uren, E., 1980. Seasonal pattern in childhood viral lower respiratory tract infections in Melbourne. Med. J. Aust. 1, 22–24.

Neubert, W.J., Eckerskorn, C., Homann, H.E., 1991. Sendai virus NP gene codes for a 524 amino acid NP protein. Virus Genes 5, 25–32.

Pumpens, P., Grens, E., 1999. Hepatitis B core particles as a universal display model: a structure–function basis for development. FEBS Lett. 442, 1–6.

Pumpens, P., Grens, E., 2002. Artificial gene for chimeric virus-like particles. In: Khudyakov, Y.E., Fields, H.A. (Eds.), Artificial DNA: Methods and Applications. CRC Press LLC, Boca Raton, pp. 249–327.

Razanskiene, A., Schmidt, J., Geldmacher, A., Ritzi, A., Niedrig, M., Lundkvist, Å., Krüger, D.H., Meisel, H., Sasnauskas, K., Ulrich, R., 2004. High yields of stable and highly pure nucleocapsid proteins of different hantaviruses can be generated in the yeast *Saccharomyces cerevisiae*. J. Biotechnol. 111, 319–333.

Rima, B.K., 1983. The proteins of morbilliviruses. J. Gen. Virol. 64, 1205–1219.

Romanos, M.A., Scorer, C.A., Clare, J.J., 1992. Foreign gene expression in yeast: a review. Yeast 8, 423–488.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning. A Laboratory Manual, 3rd ed. CSHL Press, Cold Spring Harbor/New York.

Samuel, D., Sasnauskas, K., Jin, L., Beard, S., Zvirbliene, A., Gedvilaite, A., Cohen, B., 2002. High level expression of recombinant mumps nucleoprotein in *Saccharomyces cerevisiae* and its evaluation in mumps IgM serology. J. Med. Virol. 66, 123–130.

Samuel, D., Sasnauskas, K., Jin, L., Gedvilaite, A., Slibinskas, R., Beard, S., Zvirbliene, A., Oliveira, S.A., Staniulis, J., Cohen, B., Brown, D., 2003. Development of a measles specific IgM ELISA for use with serum and oral fluid samples using recombinant measles nucleoprotein produced in *Saccharomyces cerevisiae*. J. Clin. Virol. 28, 121–129.

Sasnauskas, K., Jomantiene, R., Januska, A., Lebediene, E., Lebedys, J., Janulaitis, A., 1992. Cloning and analysis of a *Candida maltosa* gene which confers resistance to formaldehyde in *Saccharomyces cerevisiae*. Gene 122, 207–211.

Sasnauskas, K., Buzaite, O., Vogel, F., Jandrig, B., Razanskas, R., Staniulis, J., Scherneck, S., Krüger, D.H., Ulrich, R., 1999. Yeast cells allow the high-level expression and formation of polyomavirus-virus like particles. Biol. Chem. 380, 381–386.

Slibinskas, R., Samuel, D., Gedvilaite, A., Staniulis, J., Sasnauskas, K., 2004. Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*. J. Biotechnol. 107, 115–124.

Spehner, D., Kirn, A., Drillien, R., 1991. Assembly of nucleocapsidlike structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus protein. J. Virol. 65, 6296–6300.

Thorne, H.V., Dermott, E., 1976. Circular and elongated linear forms of measles virus nucleocapsid. Nature (London) 264, 473–474.

Ulrich, R., Nassal, M., Meisel, H., Krüger, D.H., 1998. Core particles of hepatitis B virus as carrier for foreign epitopes. Adv. Virus Res. 50, 141–182.

Warnes, A., Fooks, A.R., Dowsett, A.B., Wilkinson, G.W., Stephenson, J.R., 1995. Expression of the measles virus nucleoprotein gene in *Escherichia coli* and assembly of nucleocapsid-like structures. Gene 160, 173–178.

Whimbey, E., Englund, J.A., Couch, R.B., 1997. Community respiratory virus infections in the immunocompromised patiens with cancer. Am. J. Med. 102, 10–25.

Zvirbliene, A., Kucinskaite, I., Sezaite, I., Samuel, D., Sasnauskas, K., 2007. Mapping of B cell epitopes in measles virus nucleocapsid protein. Arch. Virol. 152, 25–39.