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Specificity of packaging mRNAs in bacteriophage GA virus-like particles in yeast *Saccharomyces cerevisiae*

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Abstract

Heterologous expression of RNA bacteriophage coat protein genes leads to formation of virus-like particles that encapsulate intracellular RNA. Here we packaged specific mRNA molecules into bacteriophage GA capsids *in vivo*. For specificity we employed a GA operator – a 21-nucleotide-long RNA sequence that folds into a stem-loop structure and can specifically bind to the coat protein of the phage. Several plasmids were constructed for expression in *Saccharomyces cerevisiae*, which directed synthesis of both the GA coat protein and the mRNA to be packaged. As models for packaging, three different mRNAs (GA coat protein, ENA-78 and GFP) were used, each constructed in two versions that differed by the presence of the GA operator within their sequence. RNA content analysis of the produced capsids in gel electrophoresis revealed the existence of RNAs of predicted lengths. The presence of the packaged mRNAs in the capsids was further verified by reverse transcription PCR. However, the operator had rather small effect on the specificity of capsid contents, since the mRNA of GA coat protein was also invariably detected inside the capsids.

Key words: bacteriophage GA, coat protein, mRNA, packaging, virus-like particles.

Introduction

RNA bacteriophages belonging to the Leviviridae family are small viruses that infect several Gram-negative bacteria genera. RNA phages infecting *Escherichia coli* have been divided into four groups denoted I to IV (Fiers 1979). Groups I and II are related to each other and belong to the Levivirus genus; phages from groups III and IV are also closely similar and assigned to the Allolevivirus genus. The best-known representatives are phages MS2 from group I, GA from group II, Qβ from group III and SP from group IV.

The genomic RNA of leviviruses is an approximately 3500-nucleotides-long single-stranded RNA molecule that codes for only four proteins: coat protein, lysis protein, maturation protein and a subunit of the viral replicase – an RNA-dependent RNA polymerase (for a review, see van Duin 1988). The viral genome is enclosed in a $T = 3$ quasi-equivalent icosahedral capsid with diameter about 28 nm, which consists of 180 coat protein molecules. Each virion also contains a single copy of the maturation protein, which is essential for the attachment of the phage to bacterial F-pili and successful infection.

Besides its structural role, the coat protein also functions as a specific RNA binding protein that recognizes a stem-loop structure at the very beginning of the replicase gene (Bernardi, Spahr 1973). The stem-loop is often designated a translational operator, as its binding to the coat protein effectively represses the synthesis of replicase. The operator is

also believed to be a packaging signal that initiates the assembly of the capsid and thus ensures recognition and selective encapsidation of the phage RNA (Hohn 1969a; Beckett, Uhlenbeck 1988). Although the coat protein can assemble into phage-like particles *in vitro* when mixed with the genomic RNA of the phage or unspecific RNAs of different lengths (Hohn 1969b), the presence of the operator lowers the concentration requirements for capsid assembly (Beckett et al. 1988).

The coat protein genes from numerous RNA phages have been reverse-transcribed, cloned and expressed in *Escherichia coli*, leading to assembly of virus-like particles (VLPs) without the need for any other viral components (Kastelein et al. 1983; Kozlovskaya et al. 1986; Peabody 1990; Kozlovska et al. 1993; Lim et al. 1994). Such recombinant VLPs encapsulate intracellular RNA (Pickett, Peabody 1993), but are morphologically and immunologically indistinguishable from native phages.

There has also been some interest to produce VLPs *in vivo* with a specific nucleic acid content. For this, a coexpression system that directs simultaneous production of both the coat protein and the RNA to be packaged is generally utilized. For specificity, the target RNA contains the translational operator of the replicase gene. In this way, recombinant MS2 capsids containing *LacZ* RNA were produced in *E. coli* (Pickett, Peabody 1993). A similar system was recently employed also in yeast *Saccharomyces cerevisiae* where the mRNA of human growth hormone was packaged into MS2 VLPs (Legendre, Fastrez 2005), demonstrating the potential of RNA phage capsids as carriers and possible delivery vehicles for therapeutic mRNAs. Others have made use of the remarkable stability of RNA phage capsids and engineered VLPs as containers for ribonuclease-protected RNA molecules of choice. As potential controls and standards for RT-PCR detection of RNA viruses, MS2 capsids have been produced that contain RNAs with a consensus sequence from HIV-1 *gag* gene (Pasloske et al. 1998) or, similarly, a longer RNA with sequences combined from hepatitis C virus, SARS coronavirus and avian influenza virus genomes (Wei et al. 2008).

Up to now, all efforts to encapsulate heterologous RNAs into RNA phage capsids *in vivo* have been directed towards phage MS2, a type species of group I. Since the details of molecular interactions between the coat protein and RNA operator vary among different RNA phages, the results obtained with one phage cannot be extrapolated to others. We investigated whether specific RNAs can be encapsulated *in vivo* using the coat protein from phage GA, a characteristic representative of group II.

Materials and methods

Construction of plasmids

The construction of pESC-GA has been described elsewhere (Freivalds et al. 2008). pESC-GAop was constructed by amplifying a fragment from pGACPop with primers GA_fwd and GAop_rev3 (for nucleotide sequences of primers, see Table 1), which was cloned into *Bam*HI/*Hind*III-digested pESC-URA vector (Stratagene). Note: pGACPop is an *E. coli* expression plasmid that contains the GA operator sequence downstream the GA coat protein gene, with nucleotide sequence corresponding to primers GAop_rev1, GAop_rev2 and GAop_rev3. Vectors for the two-gene coexpression plasmids were prepared by digesting pESC-GA with *Not*I and *Bgl*II (pESC-GAv1) or with *Eco*RI and *Not*I (pESC-GAv2). A short double-stranded DNA fragment containing GA operator sequence and

Table 1. Oligonucleotides used for the construction of plasmids. Restriction sites for cloning are underlined and the initiation and termination codons of genes are shown in bold

Oligonucleotide	Nucleotide sequence (5' to 3')
ENA_fwd	TCGAATTC**ATG**GCTGGTCCTGCCGC
ENA_rev	ATGCGGCCGC**TTA**GTTTTCCTTGTTTCCA
GA_fwd	CAGGATCC**ATG**GCAACTTTACGCAGTTTCGT
GA_rev	TGAAGC**TTA**CGCGTAGAAGCCACTCTG
GAop_rev1	ACATAGGTTTTCCTTATGTTTTGC**TTA**CGCGTAGAAGCCACTCTG
GAop_rev2	CATGATCAATTGACCTCCTTATCGGAACATAGGTTTTCCTTATGTT
GAop_rev3	AGAAGCTTCATGATCAATTGACCTCC
GAop1	GGCCGCAAAACATAAGGAAAACCTATGTTCCA
GAop2	GATCTGGAACATAGGTTTTCCTTATGTTTTGC
GFP_fwd	TCGAATTCC**ATG**GTGAGCAAGGGCGAGGA
GFP_rev	GAGCGGCCGCAAGC**TTA**CTTGTACAGCTCGTCCAT

*Not*I and *Bgl*II sticky ends was obtained by hybridizing GAop1 and GAop2 and ligated into pESC-GAv1. The resulting plasmid was digested with *Eco*RI and *Not*I, thus producing pESC-GAv3. A DNA fragment containing the coding sequence of ENA-78 flanked by *Eco*RI and *Not*I restriction sites was amplified from plasmid pTRC-ENA (I. Cielēns, unpublished data) using primers ENA_fwd and ENA_rev and cloned into pESC-GAv2 and pESC-GAv3, resulting in plasmids pESC-GA-ENA and pESC-GA-ENAop, respectively. In a similar manner, the sequence coding for GFP was amplified using primers GFP_fwd and GFP_rev from plasmid pA62 (kindly provided by A. Strods), which contains the gene for CXCR4-eGFP fusion protein (unpublished data). The resulting fragment was cloned into pESC-GAv2 and pESC-GAv3, thus producing plasmids pESC-GA-GFP and pESC-GA-GFPop, respectively.

Production and purification of GA VLPs

Yeast transformants were obtained, cultivated and the recombinant GA capsids purified essentially as described (Freivalds et al. 2008), with an additional last purification step of ion-exchange chromatography. After gel-filtration, the fractions containing GA VLPs in TEN buffer (20 mM Tris-HCl, pH 7.8, 5 mM EDTA, 150 mM NaCl) were loaded on a DEAE-Sephadex A50 column (V = 3.5 mL, h = 5 cm) and washed with four column volumes of TEN buffer. GA capsids were eluted in the void volume while nucleic acid contaminants remained bound to the column and were subsequently eluted with TEN buffer supplemented with 1 M NaCl. Fractions containing GA VLPs were pooled and stored frozen at –20 °C until use.

RNA extraction

An equal volume of Tris-HCl buffered phenol (pH 6.7) was added to a preparation of purified capsids, vortexed for 30 s and centrifuged at 10 000 g for 5 min. The aqueous phase was collected and repeatedly phenol-extracted until no protein band could be observed at the phase interface. The aqueous phase was then washed three times with diethyl ether and the RNA concentrated by ethanol precipitation. Finally, the RNA was dissolved in a small

volume of sterile water and aliquots stored frozen at –20 °C until use.

RNA electrophoresis

RNA samples were thawed on ice. After adding of 2X RNA loading dye (Fermentas) samples were heated at 70 °C for 10 min, cooled 2 min on ice and immediately loaded on a denaturing urea-polyacrylamide gel (8 M urea, 4 % polyacrylamide, 1X TBE). After electrophoresis, the gel was stained with ethidium bromide and RNA detected by fluorescence in UV light.

Reverse transcription PCR

Synthesis of the first strand cDNA was conducted by the RevertAid kit (Fermentas) according to manufacturer's protocol and using 1.2 μg of the extracted RNA as template and either 20 pmol of sequence-specific primer (GA_rev for all RNA samples and ENA_rev for RNA extracted from GA-ENA and GA-ENAop capsids) or 90 pmol of oligo(dT)$_{18}$ for RNA preparations from GA-GFP and GA-GFPop. The reason for using oligo(dT)$_{18}$ instead of GFP_rev was the apparent formation of a stable secondary structure of the latter, resulting in no detectable reaction products at 37 °C (data not shown). After reverse transcription, 2 μL of the mixture was used as a template for second strand cDNA synthesis, using primers GA_fwd / GA_rev, ENA_fwd / ENA_rev and GFP_fwd / GFP_rev for the amplification of GA coat protein, ENA-78 and green fluorescent protein cDNAs, respectively.

Results

Construction of the in vivo *packaging system*

To attempt to produce GA VLPs *in vivo* with a specified RNA content, we chose the expression system in yeast *Saccharomyces cerevisiae*. Although the levels of heterologous protein expression in yeast are generally lower compared to those attainable in *E. coli*, the *S. cerevisiae* system is beneficial in a number of aspects. Yeast provides a source of eukaryotic, 5'-capped and 3'-poly(A)-tailed mRNAs, which is attractive considering our further goals to test GA VLPs as RNA packaging and delivery tools to mammalian cells. Also, the absence of bacterial endotoxins in yeast preparations simplifies the capsid purification procedures for this purpose. As Legendre and Fastrez (2005) demonstrated with MS2 coat protein that such system is functional in *S. cerevisiae*, we used a similar approach for the coat protein of phage GA.

To construct the *in vivo* packaging system, we used the commercial pESC-URA plasmid, which contains divergent galactose-inducible promoters GAL1/GAL10. We have previously described the construction of pESC-GA, a pESC-URA-derived plasmid that contains the wild-type GA coat protein gene under the control of GAL1 and directs production of GA VLPs in *S. cerevisiae* cells (Freivalds et al. 2008). In order to determine the effect of GA operator on the RNA content of the VLPs, we constructed in an analogous way a plasmid pESC-GAop, which contains the GA operator sequence downstream the coat protein gene.

We proceeded to modify pESC-GA to allow the encapsidation of heterologous mRNAs of choice into GA VLPs. The system was designed in a way that any gene of interest can be inserted under the GAL10 promoter in two vectors using the same cloning sites, in which one vector (pESC-GAv3) contains the GA operator just downstream the inserted

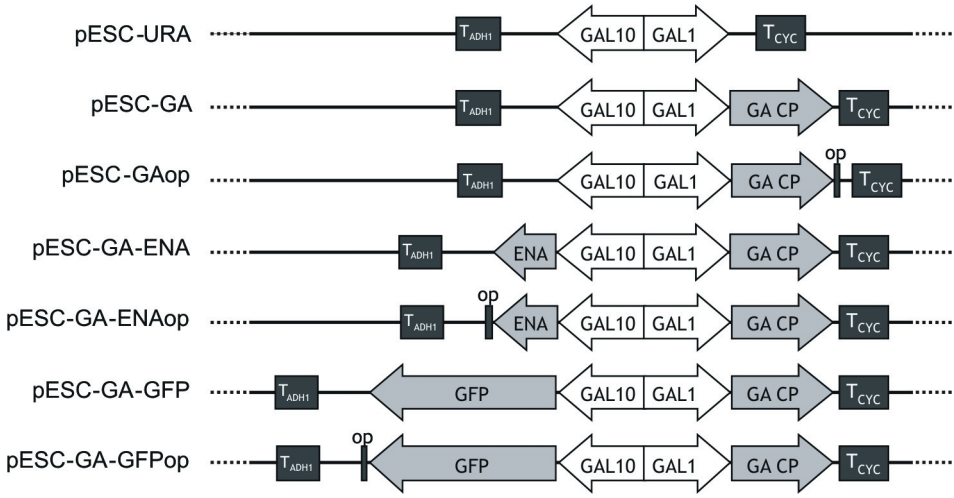

Fig. 1. Constructed plasmids for expression in S.cerevisiae. A schematic representation of the developed constructions. Genes coding for the GA coat protein (GA CP), epithelial neutrophil-activating peptide 78 (ENA) and green fluorescent protein (GFP) are indicated as well as promoters (GAL1 and GAL10), transcription terminators (T_{ADH1} and T_{CYC}) and GA operator (op).

gene, while the other (pESC-GAv2) does not and serves as a control to examine the encapsidation specificity that the operator provides. We used in the above-mentioned way two different genes to test the system: one coding for the epithelial neutrophil-activating peptide 78 (ENA-78), resulting in plasmids pESC-GA-ENA and pESC-GA-ENAop, and the other for enhanced green fluorescent protein (eGFP) with plasmids pESC-GA-GFP and pESC-GA-GFPop, respectively (Fig. 1).

Purification of GA VLPs

The purification of recombinant GA capsids from *S. cerevisiae* cells followed directly the procedures described by Freivalds et al. (2008). The fractions containing GA VLPs were remarkably pure from protein contaminants after the last step. However, electrophoretic analysis in an ethidium bromide-stained agarose gel revealed that the preparation also contains a substantial amount of free RNA that was not incorporated into capsids (data not shown). In order to adequately analyze the RNA content of the particles, removal of all other nucleic acids from the sample is of key importance. The RNA contaminants were effectively separated by introducing an additional purification step of ion-exchange chromatography on a DEAE-Sephadex A50 column. This ensured that the RNA subsequently extracted from the preparation originated only from the interior space of GA VLPs.

For convenience, the capsids produced from pESC-GA, pESC-GAop, pESC-GA-ENA, pESC-GA-ENAop, pESC-GA-GFP and pESC-GA-GFPop will further be denoted as GA, GAop, GA-ENA, GA-ENAop, GA-GFP and GA-GFPop, respectively.

Analysis of capsid RNA contents

The RNA was phenol-extracted from purified GA capsids and subjected to electrophoresis

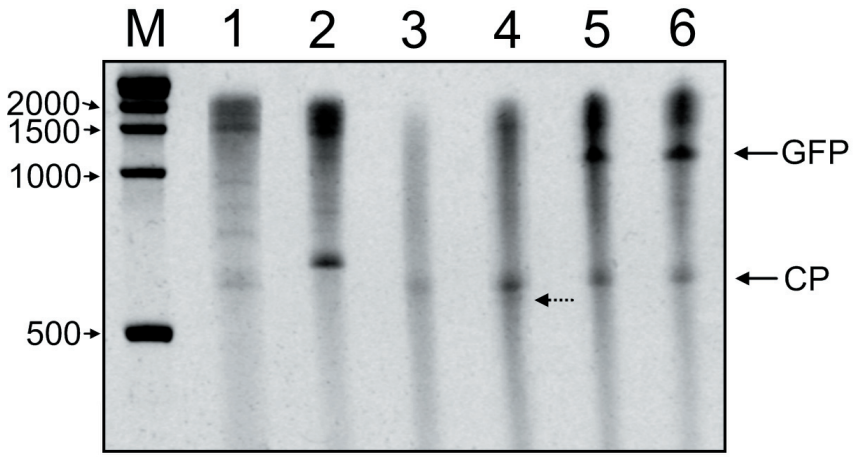

Fig. 2. Analysis of capsid RNA contents in a denaturing polyacrylamide gel. 1 μg of RNA extracted from GA (lane 1), GAop (lane 2), GA-ENA (lane 3), GA-ENAop (lane 4), GA-GFP (lane 5) or GA-GFPop (lane 6) capsids were loaded on a 4% urea-polyacrylamide gel. The positions of the GA coat protein (CP) and green fluorescent protein (GFP) mRNAs are indicated by arrows; the dotted arrow shows the supposed location of the mRNA of ENA-78. M, RNA ladder.

in a denaturing polyacrylamide gel. The RNA preparations in all cases were not homogeneous (Fig. 2). A distinct band corresponding to RNA somewhat longer than 500 nucleotides could be observed in all cases except in the preparation from GAop (lane 2), where a slightly longer RNA species was detected. Since in pESC-GAop the additional sequence with GA operator adds approximately 50 nucleotides to the 3'-untranslated region of the RNA transcript (see Table 2), this strongly suggests that the respective bands in the gel correspond to the mRNA of GA coat protein. In the case of GA-GFP and GA-GFPop (lanes 5 and 6), an additional prominent band of about 1000 nucleotides was detected, which apparently was formed by the mRNA of GFP. In the RNA preparation from GA-ENAop, a faint band could be observed just below that of the coat protein mRNA (lane 4). Further analysis in a more concentrated gel confirmed the existence of an RNA species migrating slightly faster than the mRNA of GA coat protein in preparations from GA-ENAop and also from GA-ENA, but not in those from other VLPs (data not shown). Although the sequences coding for ENA-78 and GA coat protein differ by approximately 150 nucleotides, the difference fell to about 50 nucleotides in mRNA due to unequal lengths of the untranslated regions of transcripts from GAL1 and GAL10 (Table 2). This explains the close migration and poor separation of both mRNAs and suggests that the faster-migrating band corresponds to the mRNA of ENA-78.

In order to prove unambiguously that the produced GA VLPs have packaged the expected mRNAs, the extracted RNA was analyzed by reverse transcription PCR (RT-PCR). The results (Fig. 3) showed that the mRNA of ENA-78 is present in both GA-ENA and GA-ENAop capsids (lanes 4 and 6). The mRNA of the green fluorescent protein was correspondingly present in GA-GFP and GA-GFPop capsids (lanes 8 and 10). The RT-PCR confirmed that the mRNA of the GA coat protein is present not only in GAop capsids (lane 2), but also in all other GA VLPs, regardless of the presence or absence of the operator within other mRNAs in the cell (lanes 1, 2, 3, 5, 7 and 9).

Table 2. Calculated lengths of the mRNAs packaged into the capsids. The mRNA transcription start points from GAL1 and GAL10 were determined after Johnston and Davis (1984) and the polyadenylation sites (which therefore mark the end of the 3' untranslated region) of CYC1 and ADH1 after Heidmann et al. (1992). The calculations do not include the 3' poly(A) tail, the length of which was not known but which generally spans 50-90 adenine residues in *S. cerevisiae* (Brown, Sachs 1998)

mRNA	Length of the sequence (nucleotides)			
	5'-untranslated	Protein-coding	3'-untranslated	Total
GA	66	393	126	585
GAop	66	393	178	637
ENA	12	240	280	532
ENAop	12	240	270	522
GFP	13	720	284	1017
GFPop	13	720	274	1007

Discussion

We demonstrated that it is possible to produce recombinant GA capsids *in vivo* that contain heterologous RNAs of choice. We developed a system in *S. cerevisiae* that provides a simple way to encapsulate any desired RNA sequence in GA VLPs and succeeded in packaging different model mRNAs into the particles. However, the GA operator failed to provide high encapsidation specificity of the target RNAs. This was clearly demonstrated by the incorporation of GA coat protein in mRNAs in the VLPs even in situations when other mRNAs containing the GA operator were present in the cell at the time of capsid assembly.

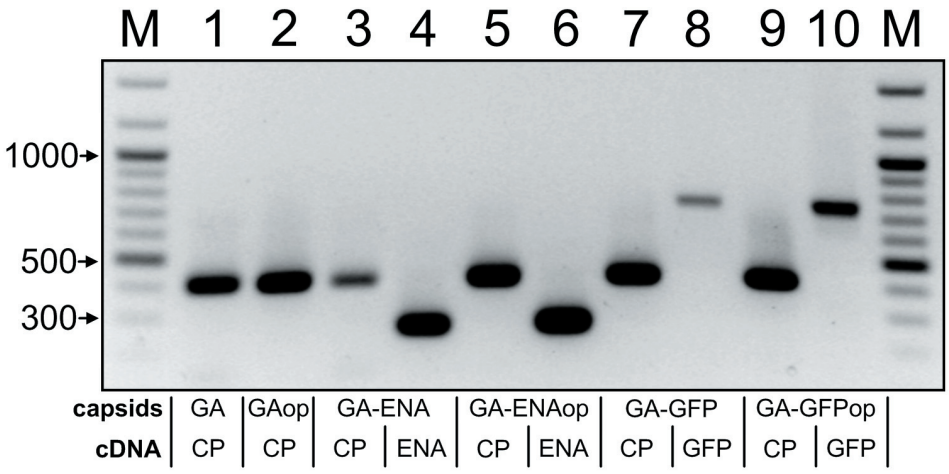

Fig. 3. Analysis of capsid RNA contents by RT-PCR. RNA was extracted from each type of the produced capsids and assayed for the presence of particular mRNAs, as indicated below. CP, cDNA of the GA coat protein; ENA, cDNA of the epithelial neutrophil-activating peptide 78; GFP, cDNA of the green fluorescent protein; M, DNA ladder.

The RNA binding properties of MS2 and GA coat proteins are slightly different. The crystal structure of the MS2 coat protein and RNA operator complex indicated that the specificity of the interaction is governed by three bases in the operator, which make direct contacts with the protein (Valegård et al. 1994). In GA this number is reduced to two, and the requirements for specific nucleotides at certain positions are also higher for MS2 than GA. MS2 coat protein only weakly binds to the GA operator, whereas GA coat protein has a similar affinity for both GA and MS2 operators (Gott et al. 1991). Consequently, GA coat protein can bind equally well to a larger pool of different RNA sequences, and the lower specificity facilitates the competition of non-operator sequences for association with the coat protein and subsequent encapsidation into the particles.

The ratio of coat protein and RNA in the cell is also important for the specificity of packaging. In a phage-infected cell, the coat protein concentration is initially low and increases gradually (Nathans et al. 1969). The high affinity of the RNA operator for the coat protein ensures specific packaging of the viral genome before the coat protein concentration reaches levels at which unspecific binding to intracellular RNA can occur (Beckett et al. 1988). The *in vivo* packaging experiments with *LacZ* mRNA showed that the specificity is indeed considerably higher at high *LacZ* and low coat protein concentrations (Pickett, Peabody 1993). The divergent GAL1/GAL10 promoters used in our system direct the production of similar levels of mRNA (Hadfield et al. 1993). In the case of MS2, this was apparently sufficient to provide a rather high specificity of packaging (Legendre, Fastrez 2005). However, to achieve high packaging specificity with GA, a higher proportion of RNA over coat protein is probably required.

For the ultimate goal to develop GA VLPs as nucleic acid delivery vehicles to eukaryotic cells, further modifications of the capsid are clearly required, which would allow to address the particles to particular types of cells. This problem might be potentially solved by chemically coupling a cell-specific peptide ligand to the surface of the capsid (Storni et al. 2004). Alternatively, chimeric RNA phage capsids can be produced by genetically fusing a foreign amino acid sequence to the coat protein (Mastico et al. 1993; Heal et al. 1999; Voronkova et al. 2002). The *in vivo* packaging system could then be used with modified coat proteins to produce chimeric capsids that contain therapeutic mRNAs or other kinds of RNA-based drugs, like ribozymes and antisense RNAs. Although the relatively low specificity of the GA coat protein-operator interaction renders the *in vivo* RNA packaging system in GA VLPs less advantageous compared to that of MS2, optimizations like adjustment of coat protein and RNA levels in the cell, the use of GA coat protein mutants that bind the operator stronger, similarly to those that are known for MS2 (Lim, Peabody 1994), and possibly other measures might significantly enhance the RNA encapsidation specificity into GA capsids in the future. Eventually, VLPs loaded with therapeutic RNAs and equipped with ligands on their surface may become powerful tools for cell-specific delivery of nucleic acid-based drugs.

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mRNS iepakošanas specifiskums bakteriofāga GA vīrusveidīgajās daļiņās raugā *Saccharomyces cerevisiae*

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Kopsavilkums

RNS bakteriofāgu apvalka proteīna gēnu heterologa ekspresija izraisa vīrusveidīgo daļiņu veidošanos, kas ietver iekššūnas RNS. Šajā darbā mēs iepakojām specifiskas mRNS molekulas bakteriofāga GA kapsīdās *in vivo*. Specifiskuma nodrošināšanai izmantojām GA operatoru – 21 nukleotīdu garu RNS sekvenci, kas salokās kāta-cilpas struktūrā un var specifiski piesaistīties fāga apvalka proteīnam. Ekspresijai *Saccharomyces cerevisiae* konstruēja vairākas plazmīdas, kas nodrošināja gan GA apvalka proteīna, gan iepakojamās mRNS sintēzi. Par iepakošanas modeļiem izmantoja trīs dažādas mRNS (GA apvalka proteīna, ENA-78 un GFP), katru no tām konstruējot divos variantos, kas atšķīrās ar GA operatora klātbūtni to sekvencēs. Iegūto kapsīdu RNS satura analīze ar gēla elektroforēzi parādīja, ka ir radušās paredzētā garuma RNS. Iepakoto mRNS klātbūtni kapsīdās apstiprināja apgrieztās transkripcijas PCR. Tomēr operatoram bija samērā niecīga ietekme uz kapsīdu satura specifiskumu, jo kapsīdās vienmēr konstatēja arī GA apvalka proteīna mRNS.