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THE COAT OF RNA PHAGE Qβ AS A CARRIER FOR FOREIGN EPITOPES

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KOPSAVILKUMS

Samērā vienkārši un labi raksturoti *Escherichia coil* RNS bakteriofāgu apvalki piesaista sev pastiprinātu pētnieku uzmanību kā svešu aminoskābju secību nesēji. RNS bakteriofāgu apvalka proteīna gēni, ekspresēti baktērijās spēcīga triptofāna promotera ietekmē, ir ideāli proteīnu inženierijas modeļi mērķtiecīgai svešo aminoskābju secību eksponēšanai. Rekombinanto RNS fāgu apvalku, kā nesēju, galvenā priekšrocība slēpjas to regulārajā struktūrā un arī augstas izšķiršanas spējas telpiskās struktūras atšifrēšanas iespējā. Dažu vairāk pētītu bakteriofāgu, tādu kā MS2, fr, GA, un Qβ, trīsdimensionālā struktūra rengenstruktūras analīzē jau ir noteikta. Pirmās seroloģiskās grupas RNS fāgu fr un MS2 rekombinantie apvalki ievadīja svešo imunoloģiski aktīvu sekvenču nesēju izveidi uz RNS fāgu bāzes. Šo apvalku galvenais trūkums ir izteikta struktūras stingrība, kas bieži nepieļāva svešu secību uzņemšanu. Lai novērstu I-ās seroloģiskās grupas RNS fāgu apvalku kapacitātes ierobežojumus, mēs izvēlējāmies III-ās seroloģiskās grupas RNS fāga Qβ klonēto apvalka proteīna gēnu par iespējamo proteīnu inženierijas modeli. Fāga Qβ, kā tipiska RNS fāgu III-ās grupas pārstāvja, priekšrocība ir 329 aminoskābju atlikumus (aa) saturošā caurlasošā apvalka, tā saucamā A1, proteīna molekulu klātbūtne kapsīdos. A1 proteīns sastāv no apvalka proteīna, kas UGA terminācijas kodona caurlasīšanas rezultātā pagarinās par 196 aa. Diemžēl, parastos apstākļos bez UGA supresijas tikai neliels A1 proteīna molekulu skaits iekļaujas apvalka sastāvā.

Darba galvenais mērķis bija, izmantojot fāga Qβ apvalku, radīt jaunu svešo aminoskābju secību nesēju. Mūsu uzdevums bija īsā apvalka proteīna C-terminālo UGA pagarinājumu, tā saukto A1-pagarinājumu, izmantot kā iespējamo svešo sekvenču lokalizācijas vietu, to eksponēšanai uz Qβ apvalka virsmas. Mēs pētījām A1-pagarinājuma novietojumu apvalkos, kā arī iespējas palielināt apvalkos iekļauto A1 proteīnu skaitu paaugstinātas UGA supresijas apstākļos.

Iegūtie rezultāti liecina par iespēju izmantot Qβ apvalka proteīnu mozaikālu vīrusveidīgu struktūru radīšanai. Mēs konstatējām, ka paaugstinātas UGA supresijas apstākļos mozaikālās Qβ daļiņās, pieaugot A1 garumam, attiecība starp apvalka proteīna normālo un pagarināto formu samazinās no 48% līdz 14%. A1 pagarinājuma novietojums uz apvalka virsmas tika pierādīts, nosakot A1 pagarinājumā iestarpinātā hepatīta B apvalka proteīna virsmas antigēna modeļ-epitopa 31-DPAFR-35 antigenitāti un imunogenitāti.

Darba rezultāti ir apkopoti 3 rakstos un ziņoti 2 starptautiskās konferencēs.

Vasiljeva, I. 1999. The coat of RNA phage Qβ as a carrier for foreign epitopes. University of Latvia, Riga, 32 p.

SUMMARY

The relatively simple and well-elucidated coat of *Escherichia coli* RNA phages had attracted considerable attention as a multimeric carriers for foreign amino acid sequences. The RNA bacteriophage coat protein genes, once expressed under the control of strong promoters in bacteria, are ideal models in protein engineering addressed to the knowledge-based introduction of foreign amino acid sequences. The principal advantage of recombinant RNA phage coat as carriers is its regularity and the availability of the highly-resolved three-dimensional structures. The three-dimensional structures of some of the more studied RNA phages, such as MS2, fr , GA, and Qβ have been determined at high resolution by X-ray crystallography. The recombinant coats of group I RNA phages fr and MS2 were first proposed as carriers for the presentation of foreign immunological epitopes. The main disadvantage of these carriers lies, however, in the fact that their marked rigidity prevents accepting of foreign amino acid sequences. To overcome the capacity limitations of the group I RNA phage coats, we offered the cloned coat protein gene of phage Qβ as a new protein engineering model. An advantage of Qβ, a member of the group III of RNA phages, is the presence of prolonged 329-aa read-through variant of coat protein, called A1 protein, consisting of coat protein and an additional 196-aa C-terminal extension separated from the coat protein sequence by an opal (UGA) stop codon. Although the A1 protein content in standard non-UGA-suppressor growth conditions averages only a few molecules to each particle.

The general problem of this study was the development of protein carrier on the basis of Qβ coat. Our task was to use the C-terminal UGA-extension of the short form of Qβ coat, so called A1 extension as a target for presentation of foreign peptides on the outer surface of Qβ particles. We investigated location of the A1 extension and the possibility to enhance the number of A1 proteins within the mosaic Qβ particles in conditions of strong UGA suppression.

Our results established the suitability of Qβ coat as a mosaic virus-like carrier. We found that in conditions of strong UGA suppression, the ratio of A1-extended to short coat proteins in mosaic Qβ particles dropped from 48% to 14%, with an increase of the length of A1-extension. The superficial location of A1-extension was demonstrated by the ensurement of specific antigenicity and immunogenicity of inserted model epitope 31-DPAFR-35 of hepatitis B surface antigen (HBsAg).

The present work is described in 3 papers and presented in 2 international conferences.

Васильева, И. 1999. Оболочка РНК бактериофага Qβ в качестве носителя чужеродных эпитопов. Латвийский Университет, Рига, 32. стр.

РЕЗЮМЕ

Относительно простые по строению и хорошо изученные оболочки РНК бактериофагов в *Escherichia coli* привлекают внимание учёных в качестве капсидоподобных носителей аминокислотных последовательностей. Гены, кодирующие белок оболочки РНК бактериофагов, экспресированные в бактериях под контролем строгого триптофанового промотора, идеальная белкого - инженерная модель для экспонирования чужеродных эпитопов. Основное достоинство РНК бактериофагов, как носителеи чужеродных последовательностей, заключается в регулярности структуры и в возможности с высокой разрешимостью определить третичную структуру. Третичная структура некоторых фагов, таких как MS2, fr, и Qβ, уже определена при помощи ренгенно структурального анализа. В качестве носителей чужеродных иммунологических эпитопов первые среди РНК фагов изучались рекомбинантные оболочки представителей I серологической групы: fr и MS2. Главный недостаток этих носителей заключается в их строго определённой структуре, недопускающей включение чужеродных аминокислотных последовательностей. Чтобы преодолеть структурные ограничения оболочки I групы РНК фагов, мы, в качестве новой белкого - инженерной модели, предложили использовать клонированный ген белка оболочки фага Qβ. Преимуществом типичного представителя фагов III серологической группы фага Qβ можно назвать присутствие удлинённого прочитываемого белка оболочки, так называемого A1 белка, длиною 329 аминокислотных последовательностей (ап). A1 белок состоит из белка оболочки удлинённого на 196 ап в С-терминальной части, отделяющей последовательность белка оболочки opal (UGA) терминирующим кодоном. К сожалению, в стандартных условиях, без UGA супрессий, только несколько молекул A1 белка включаются в состав оболочки.

Главная цель работы заключалась в создании нового носителя чужеродных аминокислотных последовательностей, используя оболочку фага Qβ. Наша задача заключалась в использовании С-терминальной UGA-удлинённой части, так называемого A1-удлинения, для экспонированния чужеродных пептидов на поверхности Qβ оболочки. Мы изучали локализацию A1-удлинения и возможность, в условиях повышенной UGA супрессии, изменить количество A1 белка в оболочке.

Наши результаты показали пригодность Qβ оболочки для создания мозаикальных носителей. Мы констатировали, что, в условиях повышенной UGA супрессии в мозаикальных Qβ частицах, соотношение между A1-удлинёнными и короткими белками оболочки падает с 48% до 14% с увиличением A1- удлинения. Поверхностная локализация A1-удлинения была доказана измерением специфичой иммуногенности и антигенности модельного эпитопа 31-DPAFR-35 гепатита В поверхностного антигена (HBsAg) внедрённого в A1-удлинение.

Настоящая работа изложена в 3 статьях и представлена на 2 международных конференциях.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to by their Roman numerals:

I Kozlovska, T., Cielens, I., **Vasiljeva, I.**, Strelnikova, A., Kazaks, A., Dislers, A., Dreilina, Dz., Ose, V., Gusars, I. & Pumpens, P. (1996). RNA phage Qβ coat protein as a carrier for foreign epitopes. *Intervirology* **39**, 9-15.

II Kozlovska, T., Cielens, I., **Vasiljeva, I.**, Bundule, M., Strelnikova, A., Kazaks, A., Dislers, A., Dreilina, Dz., Ose, V., Gusars, I. & Pumpens, P. (1997). Display vectors. II. Recombinant capsid of RNA bacteriophage Qβ as a display moiety. *Proc. Latv. Acad. Sci.* **51**, 8-12.

III **Vasiljeva, I.**, Kozlovska, T., Cielens, I., Strelnikova, A., Kazaks, A., Ose, V. & Pumpens, P. (1998). Mosaic Qβ coats as a new presentation model. *FEBS Letters* **431**, 7-11.

ABBREVIATIONS

A1	read-through coat protein of phage Qβ formed by UGA suppression of the translation termination
A2	maturation protein of phage Qβ
aa	amino acid(s)
ayw	subtype of human hepatitis B virus
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CP	short form of phage Qβ coat protein
CTL	cytotoxic T-lymphocyte
i.p.	intraperitoneally immunization
ds	double stranded
ELISA	enzyme linked immunosorbent analysis
Gag	p24 core protein of human immunodeficiency virus-1
gp120	envelope glycoprotein of human immunodeficiency virus-1
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	human hepatitis B virus
HIV	human immunodeficiency virus
I_{50}	molar amount of protein necessary for 50% inhibition of antibody binding to a solid phase
Ig	immunoglobulin
IFA	incomplete Freund's adjuvant
lac	lactose
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
preS1	polypeptide 1÷119 of the HBV gene S
preS2	polypeptide 120÷174 of the HBV gene S
RNA	ribonucleic acid
SDS	Sodium dodecyl sulfate
ss	single stranded
su^+	presence of suppression
su^-	absence of suppression
T_h	Helper T-cell
tRNA	Transport ribonucleic acid
trp	tryptophan
Ty	yeast retrotransposon
VLP(s)	virus-like particle(s)
Ty	yeast retrotransposon
VLP(s)	virus-like particle(s)

CONTENTS

1. INTRODUCTION

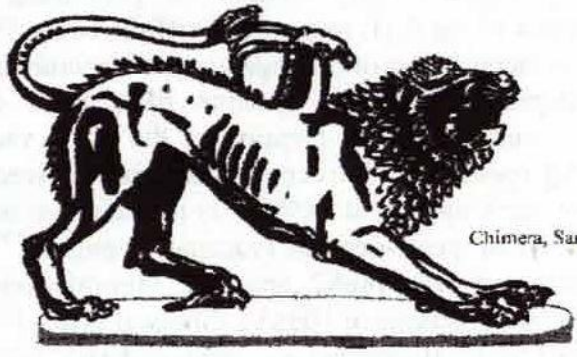
The idea of chimera, developed in the ancient Greek mythology as a monster combining elements from different animal species in its body, transformed to the molecular aspects of chimeric virus or virus-like particles (VLPs) only in the beginning of 1980s (Fig. 1). Such chimeric particles are composed of fused proteins with different parentage. In many cases, carrier portion of the fusion protein can be recognized as a body of the chimera and sequences of foreign proteins are attached at the N- and/or C-terminal parts of the carrier protein. However, internal localization of foreign sequences is also not excluded. Epitopes of viral, bacterial or other parasite proteins, nucleic-acid binding motifs and ligands for a particular receptor molecule can be inserted into carriers as foreign sequences. In contrast to the ancient chimera, these modern creatures are supposed to be useful and benevolent in molecular biology, and open a completely new route in microbiology, immunology, biomedicine and virology. The multimeric structure of such chimeras provides possibility to display remarkable amounts of foreign determinants on their surface, that makes them applicable for development of new diagnostics and vaccines, and, in future, for gene therapy as envelopes providing the transport of nucleic acid into the target cells.
Our attention was magnetized to the VLPs as carriers of foreign epitopes. In opposite to viruses, virus-like particles are non-infectious and therefore they possess no risk of infection. Such VLPs have the advantage of being easy produced and purified in different expression systems.
The general aim of this study consisted therefore in the creation of a new carrier on the basis of recombinant coat of RNA bacteriophage Qβ.

2. LITERATURE REVIEW

2.1. Chimeric virus-like particles

The first chimeric VLPs were prepared applying tobacco mosaic virus coat (Haynes et al., 1986), hepatitis B virus (HBV) surface (HBsAg) and core (HBcAg) antigens (Valenzuela et al., 1985; Clarke et al., 1987; Borisova et al., 1989, Stahl et al., 1989) and Ty p1 protein (Adams et al., 1994). More recently, the mosaic HBsAg virus-like construct was created by assembly of a pair of helper and chimeric proteins - HBsAg and HBsPolioAg (Delpeyroux et al., 1988). Since 1980s a lot of chimeras on the basis of VLPs have emerged (for review see Ulrich et al., 1998), and we already can present the three general ways of their application.
Antiviral strategy would be the first and the most significant application of VLPs including development of vaccines, serological diagnostics, gene therapy, and studies of separate sequences of the virus envelope and their role in virus life. Non-infectious is the main advantage of VLPs, taking interest of scientists in employment of them in the fight against viruses.

Chimera in ancient Greek mythology



Chimera, Sanguineto (Florence)

Chimeras in modern molecular biology

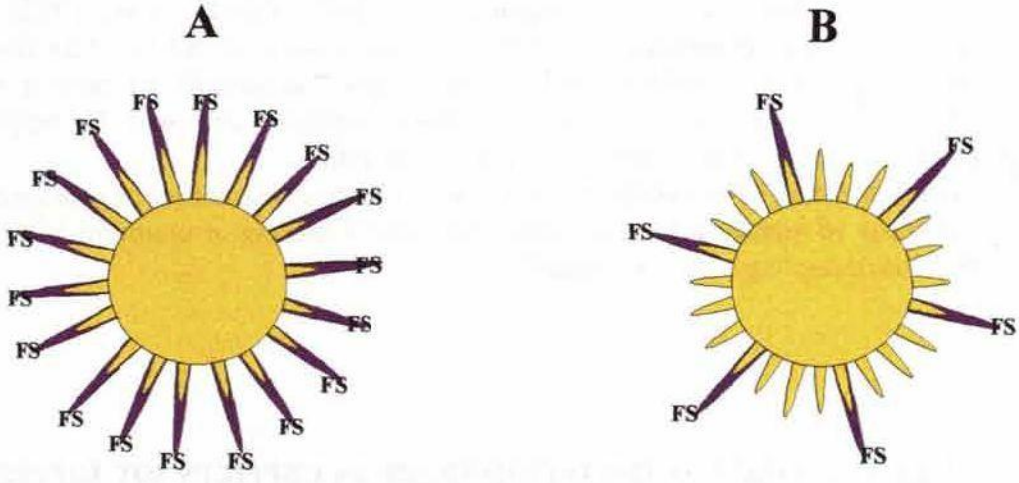


Fig. 1. Schematic presentation of the idea of the development of chimeric body from the ancient Greek mythology till present time. Chimera in modern molecular biology created on the basis of virus coat are shown: **A**, each monomer harbors insertion of foreign sequences (FS) in the particle; **B**, mosaic particles where non-mutated helper coats allow inclusion of FS-containing monomers.

VLPs as non-replicate vaccines can induce strong immune response because of correct folding of monomeric proteins as a result of formation of multimeric structures of identical subunits containing conformational or linear B cell determinants, as well as T_h and CTL epitopes (Cupps et al., 1993; Schirmbeck et al., 1996). They are often effective with adjuvants approved for use in humans, thus avoiding inadvertent side effects, and even in the absence of any additional adjuvant. This property may supplementary benefit of preventing sometimes observed inhibition by adjuvants of the CTL response (Griffiths et al., 1993; Schirmbeck et al., 1996). In attempt to increase the immunogenicity of recombinant antigens, a number of multimeric antigen presentation systems have been developed. Successful resolution of issue consisted in the creation of the HBV vaccines on the basis of recombinant HBsAg containing: 1) recombinant yeast-derived HBs and HBs preS2 antigens (for review see Cupps et al., 1993); 2) recombinant mammalian cell-derived (Shouval et al., 1994) or yeast-derived (vaccine Hepagene™) (Jones et al., 1998) HBsAg, HBs preS1, and HBs preS2 antigens. Simultaneous insertion of distinct epitopes at two different positions of HBsAg (Shouval et al., 1994; Jones et al., 1998), or into single sites of Ty p1 (Layton et al., 1993) and HIV Gag (Wagner et al., 1991; Griffits et al., 1993) demonstrate the potential of chimeric VLPs for design of multivalent vaccines.

Employment of VLPs in serological diagnostics is based on the general properties of particles: their multimeric structure and the possibility to selectively choose protein segments inserted into carrier.

Application of VLPs in gene therapy is promoted by the natural function of viral coat to bind viral nucleic acids (van den Worm et al., 1998, Johansson et al., 1998).

Utility of the chimeric VLPs as carriers for expression and purification of fused proteins would be considered as the second application area (Pogeller et al., 1996).

The third application area of VLPs provides possibility to study mechanisms of protein folding and assembly (Morikawa et al., 1991; Pushko et al., 1993). These studies are closely connected with two previous aspects of VLPs utilization. The knowledge of protein folding and assembly laws allows us to predict protein structures for construction of chimeras more precisely and will be helpful for production of artificial multifunctional proteins in future.

The main attention of scientists focused earlier on the construction of chimeric VLPs on the basis of viruses infecting eucaryotic cells. Creation of chimeric VLPs using coats of bacteriophages was not popular yet.

2.2. The coats of bacteriophages as carriers for foreign epitopes

Bacteriophages attracted attention of molecular biologists as one of the simplest exploitation objects for the presentation of foreign sequences (Table 1).

Tab.1. Bacteriophages as carriers for foreign epitopes

Structural group	Family	Genome	Way of utilization	References
1.With cubic symmetry	*Leviviridae*	ssRNA	virus-like particle	Kozlovskaia et al. (1988) Pushko et al. (1993) Mastico et al. (1996)
	Cystoviridae	dsRNA	unexplored	
	Microviridae	ssDNA	unexplored	
	Corticoviridae	dsDNA	unexplored	
	Tectiviridae	dsDNA	unexplored	
2.Filamentous and rod-shaped	*Inoviridae*	ssDNA	phage display	for review see Dunn (1996)
	Lipothrixviridae	dsDNA	unexplored	
3.Tailed	*Myoviridae*	dsDNA	phage display	for review see Dunn (1996)
	Siphoviridae	dsDNA	phage display	for review see Dunn (1996)
	Podoviridae	dsDNA	phage display	for review see Dunn (1996)
4.Pleomorphic	*Plasmaviridae*	dsDNA	unexplored	

The so-called phage display is an important technology where replication-competent filamentous bacteriophages are employed as carriers of foreign peptides (for review see Dunn, 1996). Phage display libraries can be used to study protein-protein interactions including receptor/ligand binding, enzyme substrate specificity's, and antibody epitope mapping (Lucic et al., 1998; Vaughan et al., 1998; Dunn, 1996). In addition to the widely used filamentous phages M13 and fd, tailed bacteriophage surface expression systems have now been developed (Table 1), and may be of special use for the selection of surface-display cDNA clones of random sequences (for review see Dunn, 1996). Despite of great success of the phage display as a system able to screen a large number of sequence mutants within a short time, it has also some

limitations. The inherent requirement of filamentous and tailed phages assembly acts as a limiting factor for general protein display with these phages groups (Levitan, 1998; Bothmann and Pluckthun, 1998; Dunn, 1996).
Unfortunately, VLPs of bacteriophages as carriers for foreign sequences (Kozlovskaia et al., 1988; Pushko et al., 1993; Mastico et al., 1993) are much less explored (Table 1). One attempt is known where the cloned coat protein of bacteriophage fr of *Leviviridae* family was utilized as the carrier for fine mapping of sequential epitopes (Sominskaya et al., 1992). In this case, the coat protein of bacteriophage fr lost its capability to associate into VLPs.

2.3. Virus-like particles of *Leviviridae* family

2.3.1. Leviviridae phages

The ssRNA *Leviviridae* phages were the first identified in 1961 by Loeb and Zinder. These ssRNA phages were independently isolated from sewage effluents throughout the world. They infect *Enterobacteria*, *Caulobacter* and *Pseudomonas*. *Leviviridae* phages are subdivided into *Levivirus* (serological groups I and II) and *Allolevivirus* (serological groups III and IV) genera (Table 2).

Tab.2. *Leviviridae* family

Genus	Serological Group	Representatives
Levivirus	I	fr, MS2, R17, f2, JP501, M12
	II	GA, BZ13, JP34, KU1, TH1
Allolevivirus	III	Qβ, M11, ST, TW18, VK
	IV	SP, FI, ID2, NL95, TW28

The serological groups of the *Leviviridae* phages have been distinguished by measuring the degree of inactivation by antiserum raised against one of the type species of each group (Watanabe et al., 1967, Nishihara et al., 1969).

Genetic peculiarities of the groups of *Leviviridae* confirmed their classification into *Levivirus* and *Allolevivirus* genera (Fig. 2).

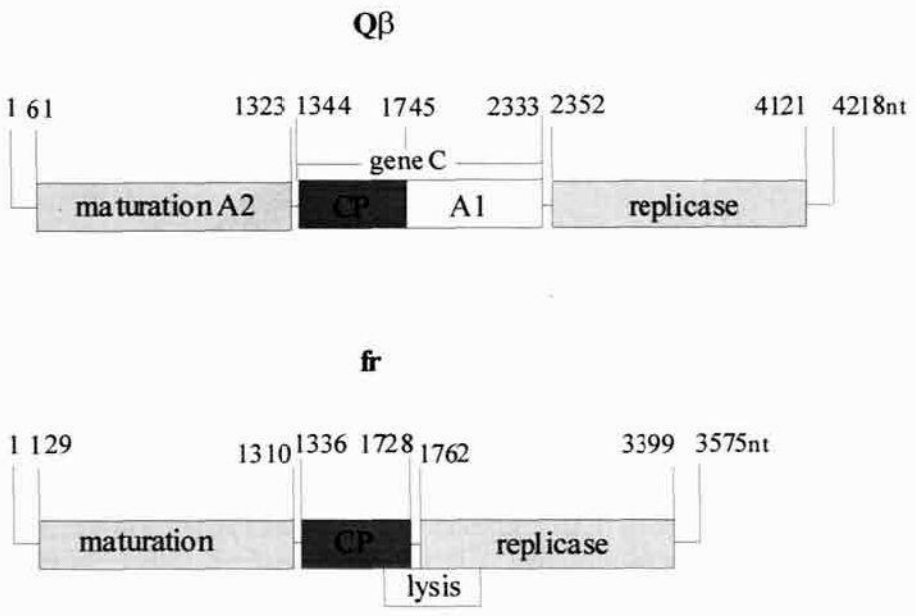


Fig. 2. Linear genome maps of bacteriophages Qβ and fr (bottom) as typical representatives of *Allolevivirus* and *Levivirus* genera, respectively.

The virion structures of *Leviviridae* are very similar. In addition to one molecule of the genomic plus strand RNA, each virion contains 180 copies of coat protein, one copy of maturation protein, and a large number of spermidine molecules that are believed to assist in the neutralization of the negatively charged genomic RNA (Fukuma and Cohen, 1975). The genus of *Allolevivirus* genus contains also the extended proteins in their coats, which are formed as the result of natural read-through of the opal (UGA) termination codon of the coat protein gene (Weiner and Weber, 1973). The diameter of the phages is 26 nm, and the protein shell is 2,3 nm wide. The icosahedral shell has a T=3 surface lattice (Zipper et al., 1971, Crowther et al., 1975). The replicase subunit and lysis protein found in the *Levivirus* genus coded for phage genome (Fig. 2) are not present in the virion.

2.3.2. Formation of virus-like particles

The first VLPs on the basis of MS2 coat protein were obtained by reassociation of the latter *in vitro* (Rohrmann and Krueger, 1970). The self-assembly of coat protein subunits of the *Levivirus* representatives MS2 (Kastelein et al., 1983) and fr (Kozlovskaia et al., 1986), and of the *Allolevivirus* genus member Qβ (Kozlovska et al., 1993) into VLPs was demonstrated also *in vivo* by expression of their genes in *E.coli*. The Ouchterlouny's double radial immunodiffusion test using anti-phage antibodies and the electron microscopy evaluation of purified recombinant products showed that the expressed genes were responsible in all cases for high-level synthesis and correct self-assembly of coat protein monomers into VLPs which were

indistinguishable morphologically and immunologically from the wild-type ones (Fig. 3).

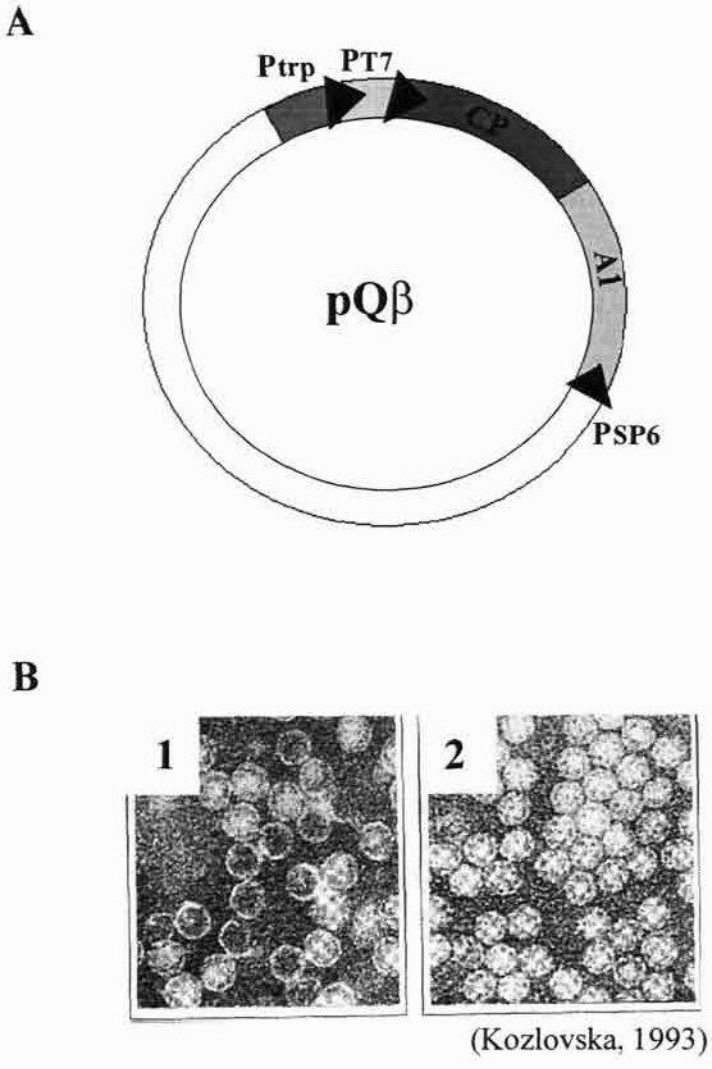


Fig. 3. Expression of the gene C of Qβ phage in *E.coli* as a typical example of creation of the VLPs. **A**, insertion of the Qβ gene C into the pGEM1 plasmid. **B**, comparison of recombinant Qβ VLPs (**1**) and native Qβ phage virions (**2**) by electron microscopy.

2.3.3. Formation of chimeric virus-like particles

The first chimeras on the basis of *Leviviridae* phages were created using VPLs of *Levivirus* representatives fr (Kozlovskaia et al., 1988; Pushko et al., 1993) and MS2 (Mastico, et al., 1993). The chimeric fr coat proteins were developed before the determination of the fr crystal structure (Liljas et al., 1994). A structure-function analysis of the capability of fr phage coat protein to assembly was undertaken using

linker-insertion, deletion and substitution mutagenesis. A series of fr coat protein variants containing sequence alterations were obtained and their assembly properties were investigated (Pushko et al., 1993). The majority of the mutants demonstrated reduction of assembly ability and formed either coat protein dimers (mutations at aa residues 2, 10, 63 or 129) or both dimer and virus-like structures (mutations at aa residues 2 or 69). The exceptions were represented by variants demonstrating normal assembly of chimeric VLPs containing (i) 19 aa insertion at position 2, (ii) 5 aa insertion at position 51, and (iii) 3 aa insertion at position 129 of the fr coat protein (Pushko et al., 1993).

The possibility to produce chimeric VLPs was studied also on the basis of phage MS2 coats (Mastico et al., 1993) whose crystal structure was the first resolved structure of RNA phages (Valegard et al., 1990). It was shown that a number of foreign peptide epitopes, up to 9 amino acids in length, could be inserted into the N-terminal hairpin between β-sheets A and B of the MS2 coat protein without significant effect on the ability of the expressed chimeric proteins to self-assemble into RNA-free VLPs *in vivo*. As expected from the external location of inserted epitopes, chimeric VLPs were immunogenic and were able to elicit specific response to the inserted sequences (Mastico et al., 1993). Unfortunately, after determination of the crystal structure of fr coats (Liljas et al., 1994), further studies of their capabilities as VLPs carriers did not appear. Little attention was demonstrated also to the phage MS2 coats as a basis for chimeric VLPs. The extremely rigid structure of icosahedrons of *Levivirus* representatives fr (Liljas et al., 1994) and MS2 (Valegard et al., 1990), and GA (Ni et al., 1996; Tars et al., 1997) prevented further development of these models.

2.4. Bacteriophage Qβ

2.4.1. Qβ virions

Qβ phage belongs to the serological group III of *Allolevivirus* genus. It was isolated originally by Watanabe (1964). As a typical representative of *Allolevivirus* genus Qβ virions are icosahedrons with quasy T=3 symmetry and consist of 180 copies of coat protein which envelop a complex of plus ssRNA molecule of 4218 bases (Horiuchi, 1975) and one copy of the maturation protein, which participates in the attachment of the phage to bacterial pili and in the lysis of bacteria (Karnik and Billeter, 1983) (Fig.2). Most of the coat monomers consist of 133aa residues, but 3 to 5 monomers are extended with an additional 196aa residues by UGA suppression of the translation termination of the coat gene. The extended coat monomers are denoted as A1 proteins. The biological function of the A1 protein and the role of read-through mechanism remain unclear yet. However, A1 molecules seem to be essential for the formation of infectious phage particles (Hofstetter et al., 1974; Engelberg-Kulka, 1977).

It was shown that the molar fraction of A1 protein in viral capsid could increase from 1.6% to 15% in relation to normal coat protein when an *E.coli* UGA suppressor strain was used as a host for propagation of Qβ phage (Weber and Koninsberg, 1975), (Fig. 4).

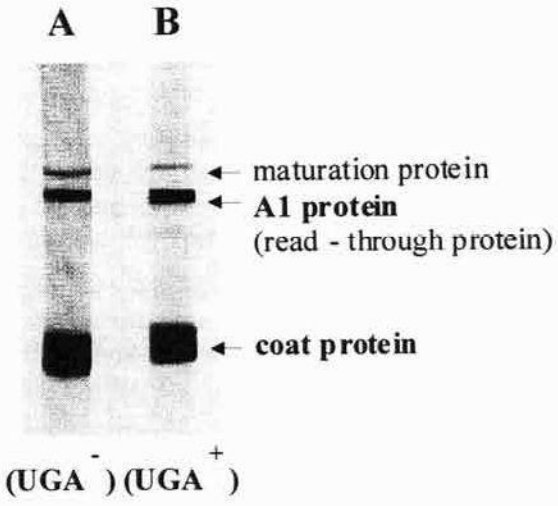


Fig.4. SDS-PAGE of Qβ virion proteins grown on su⁻ (A) and su⁺UGA (B) hosts (Zinder, 1975).

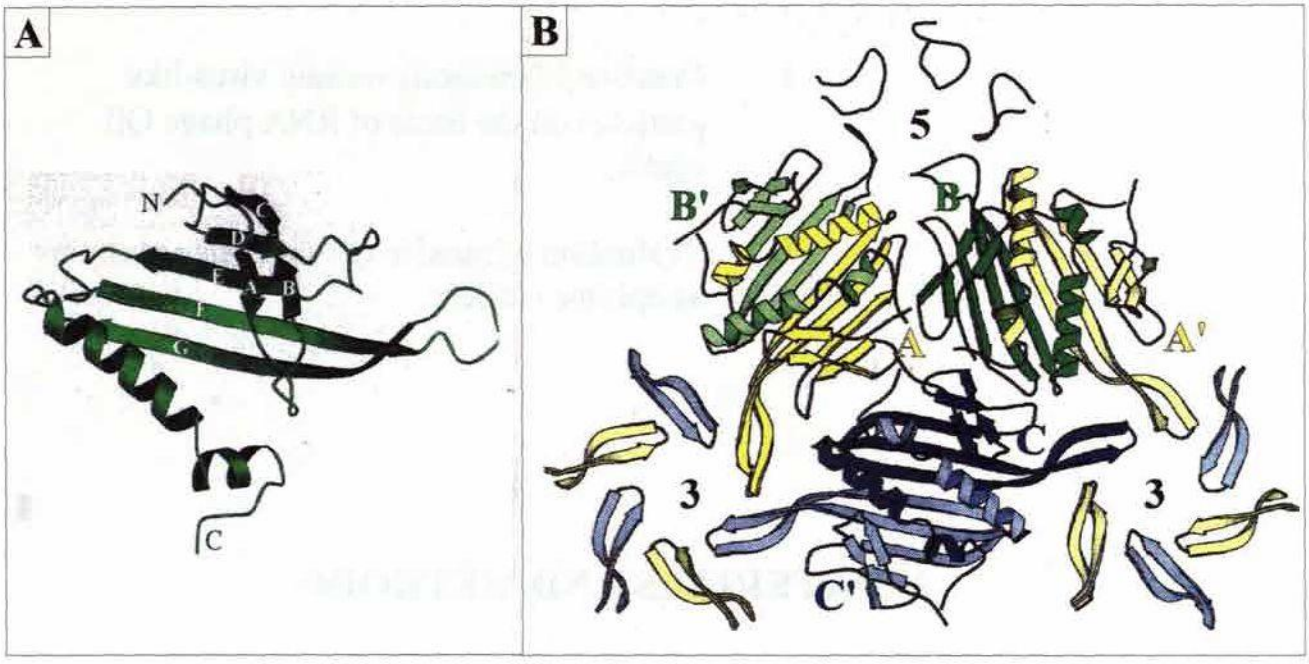
Evidence was also presented that in both UGA su⁻ and su⁺ hosts, the ratio of read-through A1 protein to normally terminated coat protein was 1,5-fold to 3-fold higher in the cells within in the purified virus. Thus, in the process of self-assembly, the viral coats prefer to incorporate the normally terminated coat monomers rather than the read-through A1 protein (Radloff and Kaesberg, 1973).

2.4.2. The crystal structure of Qβ virion

The crystal structure of the Qβ particle (Golmohammadi et. al., 1996) shows that the coat protein assumes three different conformations (denoted A, B and C) (Fig. 5). The major difference between the conformers occurs in the loop connecting the F and G β-strands (FG-loop). Similarly to *Levivirus* dimers, coat protein of Qβ forms two different types of dimers (AB and CC).

In contrast to *Levivirus* coats proteins, Qβ coat contains two cysteine residues, one at position 74 and other at position 80, which form disulfide bonds connecting adjacent FG loops. The Qβ dimers are held together in the icosahedral lattice by the disulfide bonds located around the fivefold and threefold axes. These bonds covalently connect five subunits at the fivefold axis and six subunits at the threefold axis.

The A1 protein has not been located in the electron density. This is partly due to the averaging procedure employed and to the different orientations that a coat, which is asymmetric with respect to the A1 protein, can assume within the crystal. In addition, the A1 protein could occupy positions for either one of the three subunit types: A,B or C. Supporting evidence has been presented by Takamatsu and Iso (1982), where the A1 protein was reported to be identified in both hexamers and pentamers of subunits held together by intra hexamer and pentamer disulfide bonds. In the crystal structure the C-termini of the subunits in the coat, where the read-through extension would be expected, are located at the exterior part of the coat, but are shielded from the exterior by the N-termini of the subunits with which they form dimers. A rearrangement of at least these N-termini is necessary to accommodate the extra domain (Golmohammadi et al., 1996).



(Golmahammadi, 1996)

Fig. 5. The three dimensional structure of the Qβ coat subunits. **A**, schematic drawing of a B subunit. The β sheets are denoted A-G: A (residues 17-20), B (21-27), C (32-36), D (47-53), E (62-74), and F (83-96). The two helices comprise residues 102-117 and 119-126. **B,** schematic drawing of the subunit arrangement within the Qβ particle. The three subunits A (yellow), B (green), and C (blue) representing an icosahedral asymmetric unit are shown in dark colours, and the 3 symmetry- related subunits A', B', and C' completing the dimers are shown in light colours.

2.4.3. Expression of Qβ gene C in E.coli

The phage Qβ coat protein-encoding gene C was expressed in *E.coli* under the control of strong Ptrp promoter (Kozlovska et al., 1993), (Fig. 3, part A).

Gene C contains sequences coding for both the 133-amino acid Qβ coat protein proper and the 329-aa read-through protein A1 consisting of coat protein and an additional 196-aa C-terminal sequences, separated from coat protein within the C gene by an opal (UGA) stop codon.

Expression of Qβ gene C in *E.coli* under the control of strong Ptrp promoter resulted in highly efficient synthesis of 14-kDa protein which was indistinguishable in its PAGE mobility from phage Qβ coat protein and underwent correct self-assembly into 25-nm VLPs within *E.coli* cells. Electron microscopy and Ouchterlony's immunodiffusion evaluation of these VLPs showed that they are morphologically indistinguishable from native Qβ phage particles (Kozlovska et al., 1993), (Fig. 3, part B). These Qβ particles were the first recombinant VLPs of *Allolevivirus* genus.

3. AIMS OF THE STUDY

Two general aims of the study are:

I Creation of chimeric mosaic virus-like particles on the basis of RNA phage Qβ coats.

II Evaluation of mosaic Qβ virus-like particles as epitope carriers.

4. MATERIALS AND METHODS

4.1. Antibodies

Polyclonal anti-Qβ antibodies were generated by immunization of rabbits with purified recombinant Qβ virus-like particles encoded by the UAA-terminated short form of Qβ C gene. Murine monoclonal anti-preS1 antibody MA18/7 was kindly provided by W.H. Gerlich (Giessen)

4.2. Plasmids

The basic plasmid for construction of the recombinant plasmids was pQβ containing full-length gene C of Qβ phage (Kozlovska, et al., 1993).

I - *Construction of* **pQβGGA196** *plasmid*
Two steps site-directed mutagenesis by megaprimer PCR (Barik, 1997) was used to change the UGA codon to GGA at the C-terminus of Qβ coat protein. Upstream primers: 5'-CAGCTGAACCCAGCGTACGGAACACTG-3', 5'-TAATACGACTCA CTATAGGG-3' and downstream primer: 5'-AACATCAAATTCGCGAGGCTGG-3' were synthesized and used for the synthesis of GGA-containing PCR-fragment of the Qβ C-gene that was reintroduced into the pQβ expression vector at the unique restriction sites *XbaI*, and *HindIII*.

II - *Construction of* **pQβUGA11**, **pQβUGA18**, *and* **pQβUGA24,** *and* **pQβGGA11**, **pQβGGA18**, *and* **pQβGGA24** *plasmids*
A synthetic DNA copy of the HBV preS1 sequence corresponding to 31-DPAFR-35 was inserted into plasmids pQβ and pQβGGA196 with simultaneous deletion of the downstream part of the A1 extension. Two sets of plasmids: pQβUGA and pQβGGA were created by two-step PCR. The upstream primer (5'-CAGACCGGAATTCGAG

CTCGCC-3') was the same in all of the PCR reactions. The downstream primers of the first step of PCR were 5'-GGATCGCTCGAGCCTGAGCCTCAor(TCC)ATACG CTGGGTTCAGCT-3' for construction of pQβUGA11, and pQβGGA11; 5'-GCAG GATCGCTCGAGCCTGAGCCACCACCG GCAATG-3' for pQβUGA18, and pQβGGA18; 5'-TCGAAAAGCAGGATCCGGAATAACCGGATCGGG-3' for pQβUGA24, and pQβGGA24 constructions. The products of the first PCR were used as matrices for the second PCR reactions. The downstream primer 5'-GCGCGAAG CTTATCGGAATGCAGGATCGCTCGAGCCTGAG-3' was used in the second PCR reactions for construction of pQβUGA11, pQβGGA11, pQβUGA18 and pQβGGA18, and the primer 5'-GCGAAGCTTAATTAATTATCGAAAAG CAGGATCCGG-3' for construction of pQβUGA24 and pQβGGA24. The products of the second PCR were digested with *XbaI* and *HindIII*, and cloned into the Qβ expression vector cleaved by *XbaI* and *HindIII*.

III - *Construction of* **pQβUGA203** *and* **pQβGGA203** *plasmids*
These plasmids were created by *BglII* digestion of pQβ and pQβGGA196, with subsequent insertion of a synthetic DNA copy of the preS1 sequence 31-DPAFRA-36 with sticky *BglII* ends into the *BglII* restriction site of the pQβ and pQβGGA196.

IV - *Construction of* **pQβGGA58** *plasmid*
The V3 loop copy of HIV-1 gp120 subtype MN was created by PCR on the plasmid pHBc-V3 (Grene et al., 1997) kindly supplied by G. Borisova (Latvia, LU BMC). The PCR fragment was synthesized using upstream 5'-GCGCTCCGGACATTAATTGT ACGCGTCCG-3' and downstream 5'-GCAAGCTTTAAATATTGCAATGAGCCT GA-3' primers and digested by *BspMII*, treated by Klenow fragment to generate blunt ends, and then cleaved by *HindIII* for cloning into the pQβGGA196 expression vector cleaved by *SplI*, and treated by Klenow polymerase, and cleaved then by *HindIII*.

V - *Construction of* **pQβUGA96** *and* **pQβGGA96** *plasmids*
Plasmids pQβUGA11 and pQβGGA11 cleaved by *XhoI* and *HindIII* were used as vectors. ENA-78 fragment was amplified by two steps PCR from the pRITENA-78 plasmid. Upstream 5'-GACGATGACAAGGCTGGTCCTGCCGCTGCT-3' and downstream 5'-CGCAAGCTTAGTTTTCCTTGTTTCCACC-3' primers were used for the first step of the PCR. The product of the first PCR was used as a matrix for the second PCR. The fragment synthesized by upstream primer 5'-GCGCGCTCGAGCG ATGACGATGACAAGGCTGG-3' and by downstream primer of the first PCR was cleaved then by *XhoI* and *HindIII* for cloning into the QβUGA11 and QβGGA11 vectors.

4.3. Expression and purification of mosaic Qβ particles

E.coli JM109 cells harboring the appropriate plasmids were grown overnight at 37^0C in a definite M9-based minimal medium supplemented with 10 g/l Casamino acids (Difco). Plasmid-harboring strains were stabilized by using 150 μg/ml of ampicilin or 100 μg/ml of chloramphenicol (in the case of pISM3001 plasmid).
E. coli lysates were prepared by grinding with aluminium oxide (Alcoa A-305, Serva) and ultra sonication of cells incubated in lysis buffer (10mM Tris-HCl, ph8.0, 5mM EDTA, 150mM NaCl, 0.02% TritonX-100, 2mg/ml lysozyme) during 0,5h at 4^0C. After centrifugation, proteins were precipitated from the supernatant by addition of

ammonium sulfate to 50% saturation. Pellets were resuspended in 4 ml of PBS. Chimeric Qβ particles were purified by gel filtration on Sepharose CL-4B (Pharmacia Biotech) column (1.8 x 95cm).

4.4. SDS-PAGE and Western blotting

Samples were analysed on 15% SDS-PAGE (Laemmli, 1970) stained with Coomassie brilliant blue G-250, and subjected to Western blotting in accordance to the method described by Towbin (1979).

4.5. Electron microscopy

For direct electron microscopy VLPs were adsorbed to carbon-formvar coated copper grids and stained with 2% phosphotungstic acid (ph 6.8).
Electron microscopy has been done by Velta Ose (LU BMC, Riga).

4.6. Competitive ELISA

For competitive ELISA, the synthetic peptide HBV (subtype *ayw*) preS1 p21-47 (1 μg/well) was coated onto microtiter plates and blocked overnight by 0.5% BSA (Pharmacia) at 4^0C. Wells were washed with PBS buffer containing 0.5% Tween-20. Aliquots of chimeric virus-like particles and of the p21-47 peptide as a positive control were added at varying dilutions together with the anti-preS1 antibody MA18/7 diluted to 1:15,000. The antibody dilution was chosen to yield 50% of the maximal OD_{492}, according to the calibration curve of serial dilutions of the antibody MA18/7. After 1 h incubation at 37^0C, the wells were washed, and anti-mouse Ig horseradish peroxidase conjugate (Amersham) was added. Plates were incubated for 1 h at 37^0 C, then washed, and 100 μl/well of 0.5 μg/μl o-phenylenediamine dihydrochloride (Sigma) was added. Inhibition of MA18/7 antibody binding to p21-47 peptide by the chimeric VLPs or by the control peptide p21-47 was calculated according to the following equation:
inhibition% = 100 x (1 - (OD_{492} with inhibition - bkgd) /(OD_{492} without inhibition - bkgd)), where the bkgd was the absorbance of wells coated by BSA only.
Quantitatively, the reactivity of the antigens in solution was described as I_{50}, or molar amounts of an antigen necessary for 50% inhibition of maximal antibody binding to a solid phase.

4.7. Immunization

Inbred Balb/C female mice (Grindex, Riga); (approximately 20 g in weight) were immunized by i.p. injection of 20 μg of chimeric proteins in CFA and boosted with a half-dose of proteins in IFA on the days 10 and 24.
For detection of anti-Qβ and anti- preS1 antibody titers, HBV preS1 peptide p21-47 and recombinant Qβ capsids were coated on microtiter plates at 10 μg/ml and 1 μg/ml concentration, respectively. Pooled murine sera collected on the day 24 were titrated at different dilutions. The data were expressed as antibody titer representing the highest dilution to yield three times the OD_{492} reading of preimmunization sera.

4.8. Computer analysis

The Matrix program (BioCan Scientific) was employed for determination of the ratio of A1-extended to short forms of Qβ coat protein on Western blots. The sensitive homology analysis was done by DNASTAR (Meg Align) program.

5. RESULTS AND DISCUSSION

5.1. Structural features of Qβ protein A1

Presence of A1 proteins makes the coats of *Allolevivirus* phages more attractive for peptide presentation in comparison to structurally rigid coats of *Levivirus* phages. We used sensitive homology methods to verify the exposed character and resistance to exchange of Qβ A1-extension. Since earlier experiments demonstrated existence of mixed particles of *Allolevivirus* phages Qβ and SP from serological groups III and IV with mutually exchanged A1 proteins, in spite of remarkable differences in their primary structures (Fig. 6, part A), (Priano et al., 1995), we focused our further work on alignments of Qβ A1-extension to typical protruding, superficially exposed structures such as preS sequences of HBV. In fact, Qβ A1-extension showed clear colinearity with the HBV preS sequence (Fig. 6, part B). The structural similarity of A1 extension but not of the short form of its coat protein to superficially located proteins, such as HBsAg preS1 region, especially in the specific stretch responsible for attachment of HBV virions to the hepatocytes (see for review Gerlich et al., 1992; Nassal and Schaller, 1993), suggested the putative exposure of A1-extension on the surface of Qβ phage virion.

5.2. Strategies for the synthesis of mosaic Qβ virus-like particles

The strategies allowing to realise the idea to produce the mosaic Qβ particles harboring foreign epitopes within the A1-extension are depicted on Figure 7. Here we tried to show that recombinant Qβ expression system, containing native or mutated (the UGA codon changed to UAA stop or UGG sense codons) Qβ gene C, may provide production of two kinds of mosaic particles containing homogeneous or heterogeneous insertions. Association of short coats and extended coat proteins within the particles containing homogeneous insertions is possible by expression of Qβ gene C under efficient UGA suppression. Formation of chimeric virus-like particles harboring heterogeneous insertions is more complicated. In this case expression of Qβ gene C and its derivatives could be realized from (i) the same plasmid or (ii) two plasmids conveying different antibiotic resistances to cells.

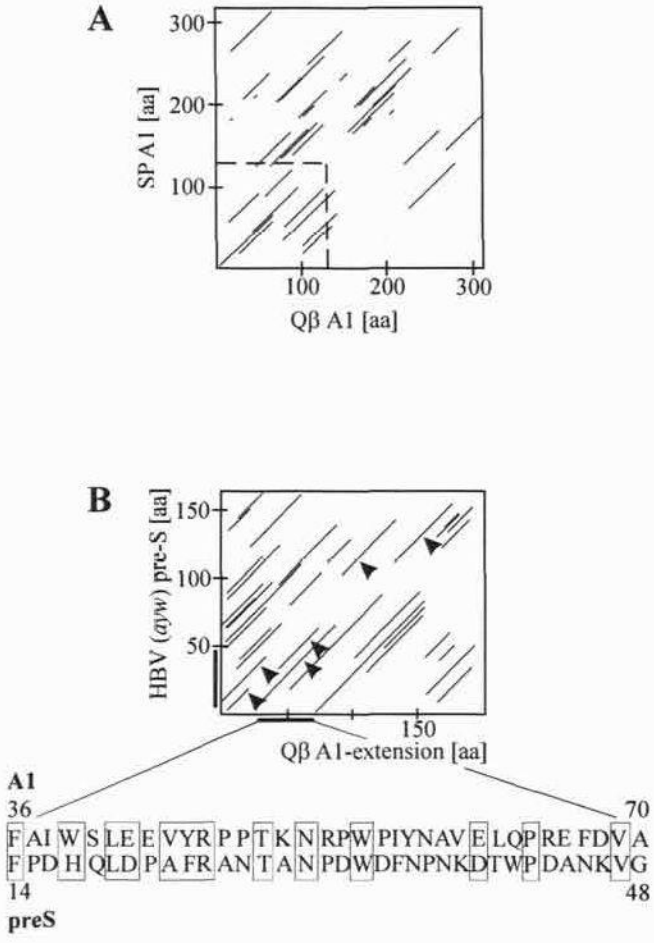


Fig. 6. Dot plot analysis of sensitive homology between Qβ, SP, and HBV preS aa sequences. **A**, sensitive homology of full length A1 protein sequences of Qβ and SP phages. Dotted line marks the end of proper CP and start of the CP extension. **B**, sensitive homology of the extension of Qβ coat protein and the HBV preS region (subtype *ayw*). Arrows indicate regions of colinear homology of CP extension and preS sequence participating in their full-length relatedness. N-terminal homologous sequences are deciphered at the bottom and reference points of homology are boxed.

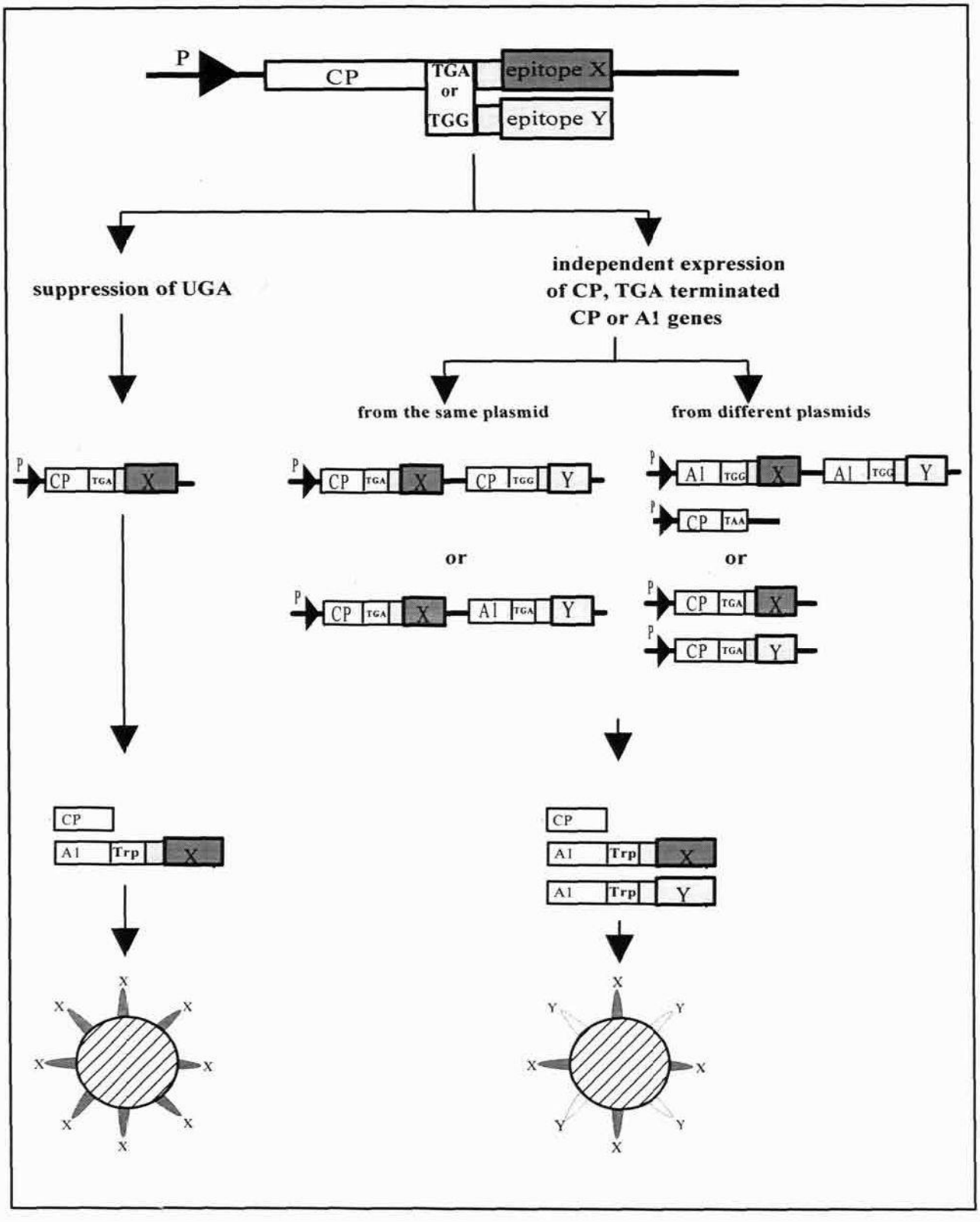


Fig. 7. Principal scheme for generation of mosaic particles carrying exposed foreign epitopes X, and Y in *E.coli* on the basis of gene C of RNA phage Qβ.

We chose the natural way of expression of Qβ gene C under the UGA suppression conditions for production of chimeric virus-like particles. Expression of gene C under the UGA suppression conditions was the first step to investigate the assembly properties of short and extended coat proteins.

The cloned full-length Qβ gene C located under the control of promoter of *E.coli trp* operon and of T7 phage (Kozlovska et al., 1993) was used as the initial plasmid for construction of stable producers of mosaic virus-like particles. Two sets of vectors (Fig. 8) were constructed with the aim to test the capacity of Qβ coat to accept extended A1-proteins. Vectors of UGA series were designed for generation of mosaic particles consisting of modified A1 proteins synthesized under UGA suppression conditions and the short coats. The other set of vectors of GGA series was constructed for generation of particles containing full-length or shortened A1 proteins only, without short coats as helpers. In this case, site-directed mutagenesis by megaprimer PCR was used to change the stop UGA codon of the coat protein to the sense GGA codon.

A synthetic DNA copy of the HBV preS1 epitope 31-DPAFR-35, corresponding to the minimal sequence that is necessary and sufficient to be recognized by the monoclonal virus-neutralizing anti-preS1 antibody MA18/7 (Sominskaya et al., 1992), was introduced as an immunomarker at different distances from the C-terminus of the short form of Qβ coat protein. This immunomarker sequence allowed us to test the location of the A1-extension into the mosaic particle. Some longer epitopes for example, the HIV-1 gp120 V3 loop subtype MN (Goudsmit et al., 1988) of 39 amino acids, and the human neutrophil-activating peptide (ENA-78) of 78 amino acids (Chang et al., 1994) were also explored (Fig. 8).

5.3 Assembly of modified A1 proteins

Capsid-forming ability of modified A1 proteins was tested in *E.coli* by expression of plasmids of pQβGGA series (Fig.8).

Obtained results showed that the native A1 protein (196 aa extension) and its full-length form with the DPAFR insertion (203 aa extension) as well as derivatives containing ENA-78 (96 aa extension) and gp120 V3 loop (58 aa extension) insertions were insoluble and formed inclusion bodies within the cells, whereas shortened A1 variants with the 24, 18, and 11 aa residue extensions were soluble and appeared as asymmetric multimeric particles that differed strongly in size and shape from icosahedral phage-like shells. Spatial limitations may explain why we found that not only full-length A1 proteins are insoluble, but also why short 11 aa residue extension is sufficient to block self-assembly of Qβ coat. These results suggested that the presence of the short form of Qβ coat protein as a helper is necessary for formation of dimers and, finally, of chimeric virus-like particles.

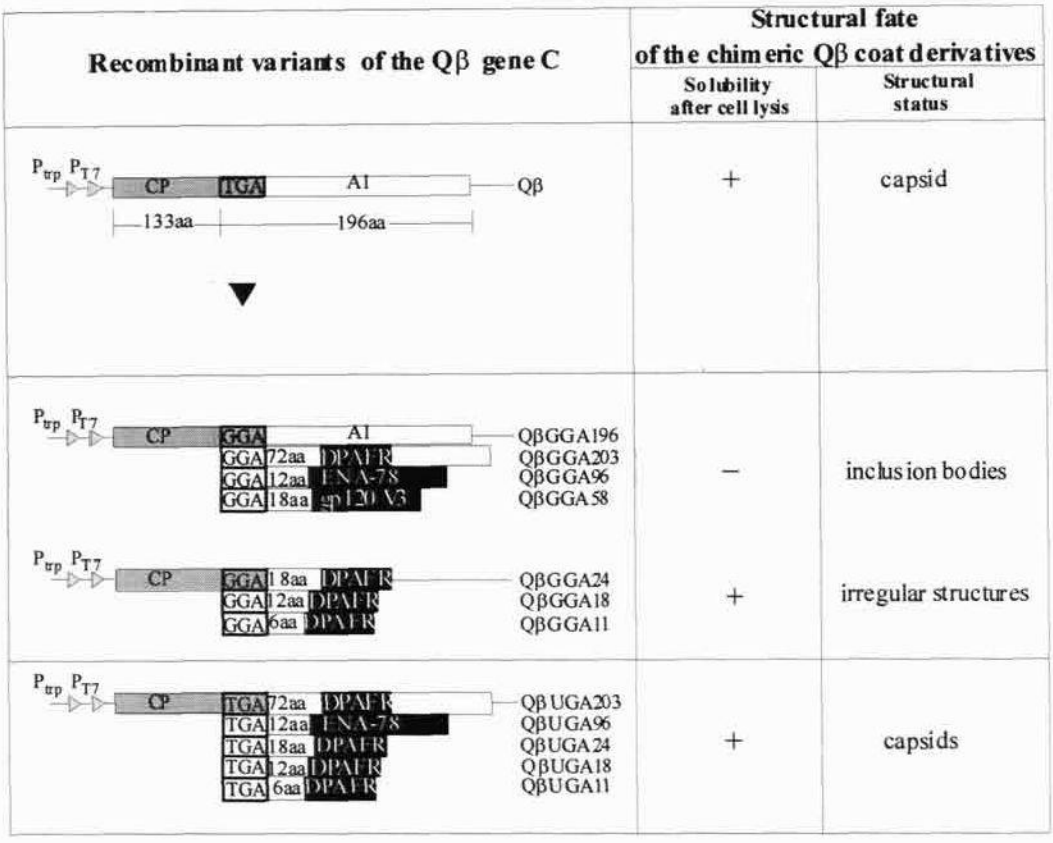


Fig. 8. Chimeric derivatives of the Qβ gene C-encoded proteins and their ability to self-assemble. The structure of the initial pQβ plasmid is shown above. Presence of inclusion bodies within the cells and capsid forming ability of Qβ coat derivatives were detected by electron microscopy.

5.4. Formation of mosaic virus-like Qβ particles

5.4.1. Enhancement of A1 protein synthesis

To enhance the expression of the A1-extended read-through genes (Qβ gene C variants) and, therefore, to enhance the content of the appropriate A1-extended proteins in mosaic particles, we used the plasmid pISM3001 which harbors cloned opal tRNA gene under the control of *lac* promoter and enables translation of UGA codon as a sense tryptophan codon (Smiley and Minion, 1993). The plasmids of the pQβUGA series (Fig. 8) were transformed into *E.coli* JM109 cells together with pISM3001 plasmid, creating strong UGA suppression conditions. Quantitative analysis of Western blots of SDS-PAGE of crude SDS cell lysates (Fig. 9, part B) demonstrated that the UGA suppression level was stable in all of the analyzed subclones, and the synthesis of A1-extended read-through proteins varied from 47% to 60% independently of the size of A1-extension.

5.4.2. Formation of mosaic particles

The products of all mentioned Qβ gene C variants assembled in particles that were purified by gel filtration on a CL4B Sepharose column. Electron microscopy revealed their icosahedral form and morphological similarity to native Qβ virions and to recombinant Qβ shells (Fig. 10). Quantitative Western blot analysis of the ratio of short to A1-extended forms of coat proteins within the particles (Fig. 9, part B) showed that these particles were in fact mosaic, and that the percentage of A1-extended coats reached 14% or 15% for a 203 or 96 aa extensions, but the content of A1-extended coats increased to 47-48% for the shorter 11, 18, or 24 aa extensions. The percentage of A1-extended proteins in the cells and in the purified particles fits well in the case of A1 derivatives containing 11 to 24 aa extensions what indicates the law of any self-assembly advantage for short coat protein in these cases. In contrast, long A1 derivatives containing 96 to 203 aa extensions were disadvantageous for self-assembly to short coat protein by a factor of about 3.

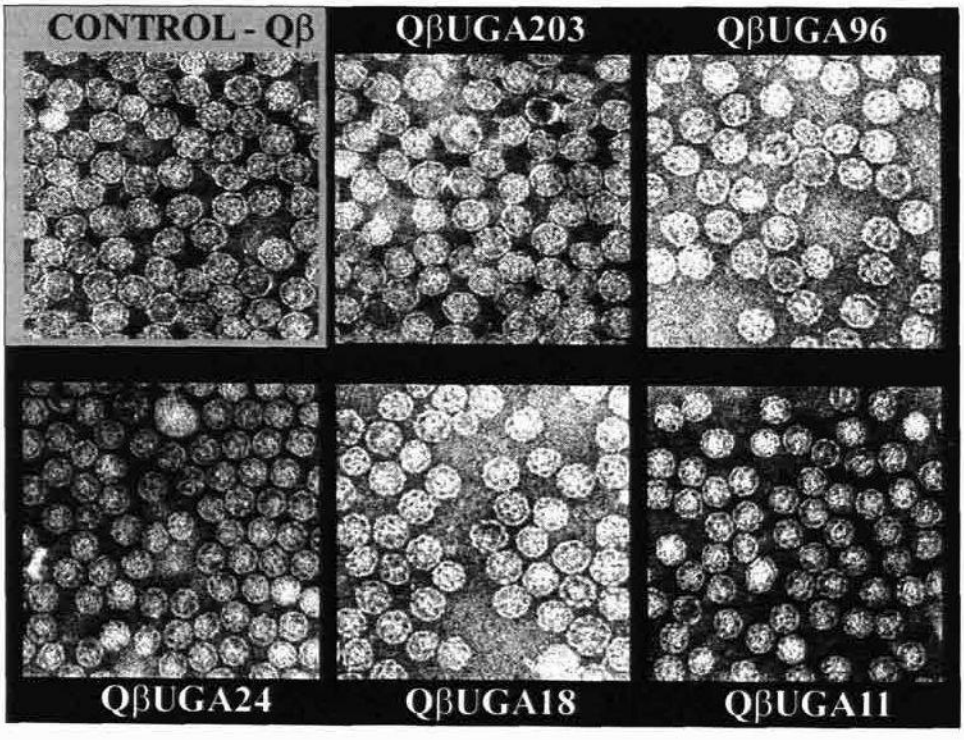


Fig. 10. Negative staining of mosaic particles purified from *E.coli* cells after expression of the appropriate A1-extended genes under strong UGA suppression conditions. Magnification 200.000 x.

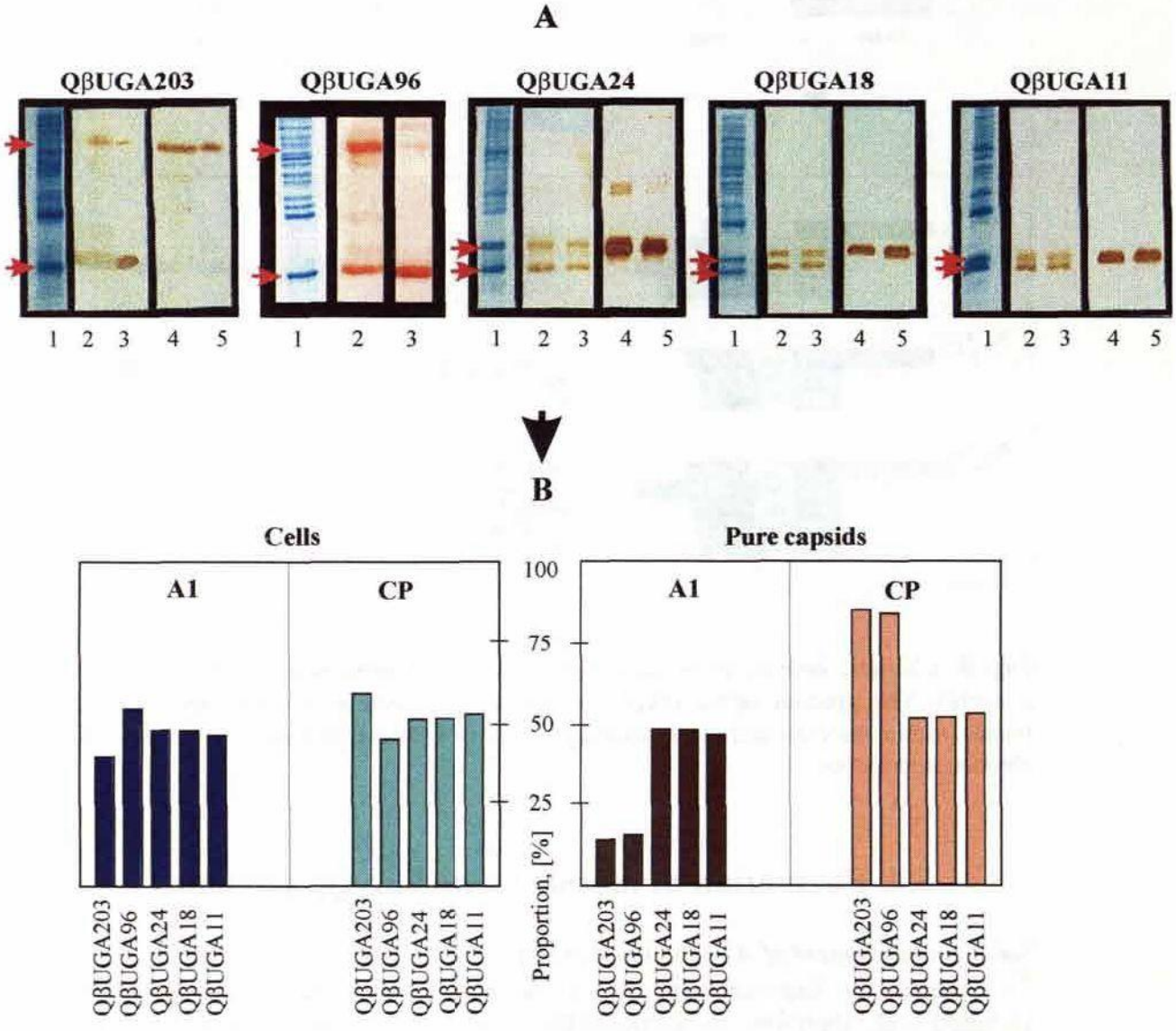


Fig. 9. The influence of Qβ coat protein extension on the ratio of CP and A1 (CP + extension [aa]) proteins in the crude *E.coli* SDS lysates (part **A**, lines 1,2,4) and pure particles (part **A**, lines 3,5) propagated by plasmids of pQβUGA series in the *E.coli* cells under efficient UGA suppression conditions: **A**, SDS-PAGE (line 1), Western blotting with polyclonal anti-Qβ antibodies (lines 2,3) and with monoclonal anti-preS1 antibody MA18/7 (lines 4,5); **B**, percentage of extended A1 and short CP proteins in *E.coli* cells (left) and within the pure capsids (right).

5.4.3. Localization of the A1-extension

In the competition ELISA test, the particles keep their structures stable what is sufficient for investigation of localization of A1-extension within the mosaic particle. We employed the competitive ELISA test of purified VLPs QβUGA203, QβUGA24, QβUGA18, and QβUGA11 with monoclonal anti-preS1 antibody MA18/7 (Fig. 11, part A) for identification of the preS1 epitope within the particle. Obtained results indicated that at least 5% of all particle proteins were A1 proteins with the 203 aa extension and 27-33% where A1 proteins in the case of 11, 18, or 24 aa extensions, the C-terminus of which was located on the surface of mosaic particle and was accessible to anti-preS1 antibody MA18/7.

5.4.4. Immunogenicity of mosaic particles

The immunogenicity of mosaic QβUGA203, QβUGA24, QβUGA18, QβUGA11 particles was tested in Balb/C mice, and their ability to induce anti-preS1 antibody response was detected by standard ELISA test (Fig. 11, part B). The inserted preS1 epitope showed remarkable immunogenicity in mice, in spite of its minimal length. Immunogenicity of the preS1 insertion increased with the reduction of the length of A1 extension. The highest immunogenicity was found for mosaic particles harboring A1 proteins with only 11 aa extension. The titer of specific anti-preS1 antibodies reached 1:3,600 in this case. The immunogenicity of mosaic particles containing A1 proteins with 18 or 24 aa extensions was similar at a level of up to 1:2,000 titer of anti-preS1 antibodies. The product containing the longer A1-extension of 203 aa was able to induce the 1:700 titer of anti-preS1 antibodies.

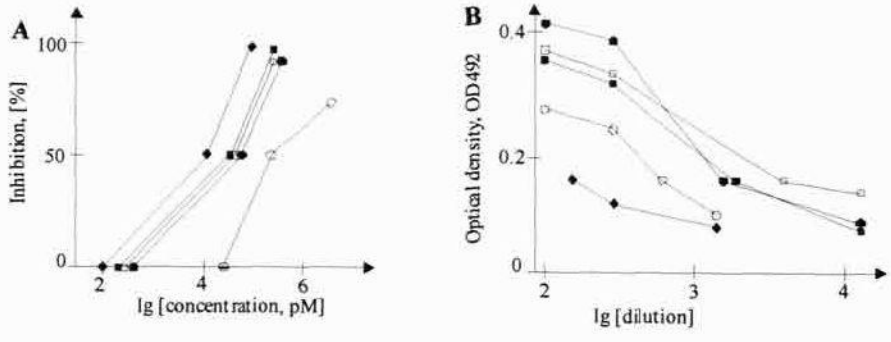


Fig. 11. Antigenic (**A**) and immunogenic (**B**) properties of the preS1 epitope 31-DPAFR-35 exposed on the surface of mosaic Qβ particles. **A,** Competition ELISA with monoclonal anti-preS1 antibody MA18/7 (**O** - QβUGA203, ● - QβUGA24, ■ - QβUGA18, □ - QβUGA11, ◆ - peptide preS1 (21-47) as a positive control). **B,** Immunological response of Balb/C mice to the preS1 epitope on day 24 (**O** - QβUGA203, ● - QβUGA24, ■ - QβUGA18, □ - QβUGA11, ◆ - mice immunized with original recombinant Qβ particles without any insertions).

5.5. Mosaic Qβ coat as one of the presentation models

The mosaic structures as one of the models of molecular presentation are little explored in molecular biology yet. These mosaic carriers as all things in the world have their advantages and disadvantages that define their application. The possibility to increase the remarkably insertion capacity belongs to the prime advantages of mosaic particles. Decrease of the ratio of extended proteins to the proper protein within the mosaic particles in parallel to an increase of the length of extension would be recognized as a disadvantageous factor limiting their application area. These principles of formation of mosaic structures are clearly demonstrated here on the basis of new carrier represented by the Qβ coats. It can be located between two existing mosaic VLPs models: HBsAg (Delpeyroux et al., 1988) and HBcAg (Koletzki et al., 1997). Increase of insertion size till 144 aa in mosaic HBcAg particles (Koletzki et al., 1997) and till 203 aa in Qβ mosaic particles was demonstrated by exploitation of the same idea of C-terminal UGA read-through extension.

The idea of mosaic structures was successfully employed for replication-competent virus carriers. The first virus mosaic particles have been constructed on the basis of the filamentous coats of DNA bacteriophages of the *Inoviridae* family and of the icosahedral coats of cowpea mosaic virus, a representative of RNA plant viruses (Dunn, 1996; Porta et al., 1996). Application of mosaic technology allowed to enhance capacities of the gene VIII- encoded major protein of filamentous phage fd from 12 to 36 aa residues (Greenwood et al., 1991). Introduction of a modified gene III-encoded minor protein of filamentous phages having a capacity of approximately 57 aa residues may be evaluated as a special case of generation of mosaic particles (Dunn, 1996). The number of gene III products within the filamentous phage is constant and does not exceed 5 copies (Model and Russel, 1988), whereas mosaic Qβ particles can include about 25 to 86 copies of A1-extended proteins per shell. The capsid of the popular cowpea mosaic virus can include 50% of the small proteins with 27 aa insertion (Porta et al., 1996).

In comparison with the existing mosaic carriers, our results demonstrate validity of mosaic Qβ virus-like particles as carriers for homogeneous insertions at the C-terminus of the extended coats.

6. CONCLUSIONS

1. Virus-like Qβ particle has been suggested as a new carrier for presentation of foreign epitopes.

2. The presence of short Qβ coat protein as a helper is necessary for formation of mosaic Qβ virus-like particles harboring insertions at the C-terminus of the A1-extension.

3. Expression of modified Qβ C genes under efficient UGA suppression conditions endow the formation of mosaic Qβ virus-like particles containing insertions at the C-terminus of A1-extension.

4. The proportion of A1-extended to short coats in mosaic Qβ virus-like particles drops from 48% to 14% with an increase of the length of the A1- extension.

5. The preS1 epitope 31-DPAFR-35 inserted into the A1-extension at the varying distances from the C-terminus of Qβ CP is located on the surface of mosaic Qβ VLPs.

6. The immunogenicity of the HBV preS1 epitope 31-DPAFR-35 inserted into the A1-extension of mosaic Qβ virus-like particles increased with the reduction of the length of the A1-extension. Titer of anti-preS1 grew from 1:700 to 1:3,600 for A1-extensions of 11 aa residues (QβUGA11) and 203 aa residues (QβUGA203), respectively.

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8. REFERENCES

Adams, S., Burns, N., Layton, G. & Kingsman, A. (1994). Hybrid Ty virus-like particles. *Int. Rev. Immunol.* **11**, 133-141.

Barik, S. (1997). Mutagenesis and Gene Fusion by Megaprimer PCR. In *PCR Cloning Protocols* (White, B. ed.), pp. 173-182. Human Press, Totowa, New Jersey.

Borisova, G., Berzins, I., Pushko, P., Pumpen, P., Gren, E., Tsibinogin, V., Loseva, V., Ose, V., Ulrich, R., Siakkou, H. & Rosental, H. (1989). Recombinant core particles of hepatitis B virus exposing foreign antigenic determinants on their surface. *FEBS Lett.* **259**, 121-124.

Bothmann, H. & Pluckthun, A. (1998). Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat. Biotechnol.* **16(4)**, 376-380.

Chang, M., McNinch, J., Basu, R. & Simonet, S. (1944). Cloning and characterization of the human neutrophil-activating peptide (ENA-78) gene. *J. Biol. Chem.* **269 (41)**, 25277-25282.

Clarke, B., Newton, S., Carroll, A., Francis, M., Appleygard, G., Syred, A., Higfield, P., Rowlands, D. & Brown, F. (1987). Improved immunogenicity of peptide epitope after fusion to hepatitis B core protein. *Nature* **330**, 381-384.

Crowther, R., Amos, L. & Finch, J. (1975). Three-dimensional image reconstructions of bacteriophages R17 and f2. *J. Mol. Biol.* **98(3)**, 631-635.

Cupps, T., Tibbles, J., Hurni, W., Miller, W., Ellis, R., Milich, D. & Wetter, N. (1993). In vitro T cell immune responses to the preS2 antigen of the hepatitis B virus envelope protein in preS2+S vaccine recipients. Absence of cross-reactivity of subtypes at a major T cell recognition site. *J. Immunol.* **151(6)**, 3353-3360.

Delpeyroux, F., Peillon, N., Blondel, B., Crainic, R. & Streeck, R. (1988). Presentation and immunogenicity of the hepatitis B surface antigen and a poliovirus neutralization antigen on mixed empty envelope particles. *J. Virol.* **62(5)**, 1836-1839.

Delpeyroux, F., van Wenzel, E., Blondel, B. & Crainic, R. (1990). Structural factors modulate the activity of antigenic poliovirus sequences expressed on hybrid hepatitis B surface antigen particles. *J. Virol.* **64**, 6090-6100.

Dunn, S. (1996). Phage display of proteins. *Curr. Opin. Biotechnol.* **7**, 547-553.

Engelberg-Kulka, H., Dekel, L. & Israeli-Reches, M. (1977). Streptomycin-resistant *Escherichia coli* mutant temperature sensitive for the production of Qβ-infective particles. *J. Virol.* **21(1)**, 1-6.

Fukuma I. & Cohen S. (1975). Polyamines in bacteriophage R17 and its RNA. *J. Virol.* **16**, 222-227.

Furuse, K. (1987) Distribution of coliphages in the environment: general considerations. In *Phage ecology* (Goyal, S., Gerba, P. & Bitton, G. eds.), Wiley @ Sons.

Gerlich W., Heermann, K. & Lu, X. (1992). Function of hepatitis B surface proteins. *Arch. Virol. Suppl.* **4**, 129-132.

Golmohammadi, R., Fridborg, K., Bundule, M., Valegard, K. & Liljas, L. (1996). The crystal structure of bacteriophage Qβ at 3.5A resolution. *Structure* **4**, 543-554.

Goudsmit, J., Debousk, C., Meloen, R., Smit, L., Bakker, M., Asher, D., Wolff, A., Gibbs, C. & Gajdusek, D. (1988). Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimmentally infected chimpanzees. *Proc. Nat. Acad. Sci. USA*, **85,** 4478-4482.

Greenwood, J., Willis, A. & Perham, R. (1991). Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from *Plasmodium falciparum circumsporozoite* protein as antigens. *J. Mol. Biol.* **220(4)**, 821-827.

Grene, E., Mezule, G., Borisova, G., Pumpens, P., Bentwich, Z. & Arnon, R. (1997). Relationship between antigenicity and immunogenicity of chimeric hepatitis B virus core particles carrying HIV Type 1 epitopes. AIDS Res. Hum. Retroviruses **13(1)**, 41-51.

Griffiths, J., Harris, S., Layton, G., Berrie, E., French, T., Burns, N., Adams, S. & Kingsman, A. (1993). Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: Induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. *J. Virol.* **67**, 3191-3198.

Haynes, J., Chang, D. & Consigli, R. (1993). Mutations in the putative calcium-binding domain of polyomavirus VP1 affect capsid assembly. *J. Virol.* **66**, 5232-5241.

Hofsetter, H., Monstein, H. & Weismann, C. (1974). The readthrough protein A1 is essential for the formation of viable Qβ particles. *Biochym. biophys. Acta* **374**, 238-251.

Horiuchi, K. (1975). Genetic studies of RNA phages. In *RNA phages* (Zinder, N. ed.), pp. 29-50. Cold Spring Harbour Laboratory, USA.

Jagadish, M., Hamilton, R., Fernandez, C., Schoof, P., Davern, K., Kalnins, H., Ward, C. & Nisbet, I. (1993). High level production of hybrid potyvirus-like particles carrying repetitive copies of foreign antigens in *Escherichia coli. Biotechnology (N Y)* **11(10)**, 1166-1170.

Johansson, H., Dertinger, D., LeCuyer, K., Behlen, L., Greef, C. & Uhlenbeck, O. (1998). A thermodinamic analysis of the sequence-specific binding of RNA by bacteriophage MS2 coat protein. *Proc. Natl. Acad. Sci. USA* **95(16)**, 9244-9249.

Jones, C., Page, M., Bacon, A., Cahill, E., Bentley, M. & Chatfield, N. (1998). Characterisation of the T- and B-cell immune response to a new recombinant pre-S1, pre-S2 and SHBs antigen containing hepatitis B vaccine; evidence for superior anti-SHBs antibody induction in responder mice. *J. Virol. Hepat.* **2**, 5-8.

Karnik, S. & Billeter, M. (1983). The lysis function of RNA bacteriophage Qβ is mediated by the maturation (A2) protein. *EMBO J.* **2**, 1521

Kasteilein, R., Berkhout, B., Overbeek, G. & Duin, J. (1983). Effect of the sequences upstream from the ribosome-binding site on the yield of protein from the cloned gene for phage MS2 coat protein. *Gene* **23(3)**, 245-254.

Koletzki, D., Zankl, A., Gelderblom, H., Meisel, H., Dislers, A., Borisova, G., Pumpens, P., Kruger, D. & Ulrich, R. (1998). Mosaic hepatitis B virus core particles allow insertion of extended foreign protein segments. *J. Gen. Virol.* **78**, 2049-2053.

Kozlovskaia, T., Pumpen, P., Dreilinia, D., Tsimanis, A. & Ose, V. (1986). Formation of capsid-like structures as a result of expression of the cloned gene of the envelope protein of the RNA-containing bacteriophage fr. *Dokl. Akad. Nauk. SSSR* **287(2)**, 452-455.

Kozlovskaia, T., Pushko, P., Stankevich, E., Dreimane, A., Sniker, D., Grinstein, E., Vejina, A., Ose, V., Pumpen, P. & Gren, E. (1988). Genetically engineered mutants of the envelope protein of the RNA-containing bacteriophage fr. *Mol. Biol. (Mosk.)* **22**, 731-740.

Kozlovska, T., Cielens, I., Dreilinna, D., Dislers, A., Baumanis, V., Ose, V., Pumpens, P. (1993). Recombinant RNA phage Qβ capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene* **137**, 133-137.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Layton, G., Harris, S., Gearing, A., Hill-Perkins, M., Cole, J., Griffits, J., Burns, N., Kingsman, A. & Adams, S. (1993). Induction of HIV-specific cytotoxic T lymphocytes *in vivo* with hybrid HIV-1 V3: Ty-virus-like particles. *J. Immunol.* **151**, 1097-1107.

Levitan, B. (1998). Stochastic modeling and optimization of phage display. *J. Mol. Biol.* **277(4)**, 893-916.

Liljas, L., Fridborg, K., Valegard, K., Bundule, M. & Pumpens, P. (1994). Crystal structure of bacteriophage fr capsids at 3.5A resolution. *J. Mol. Biol.* **244**, 279-290.

Loeb T. & Zinder, N. (1961). A bacteriophage containing RNA. *Proc. Nat. Acad. Sci. U.S.A.* **47**, 282.

Lucis, M., Forbes, B., Grosvenor, S., Carr, J., Wallace, J. & Forsberg, G. (1998). Secretion in *Escherichia coli* and phage-display of recombinant insulin-like growth factor binding protein-2. *J. Biotechnol.* **61(2)**, 95-108.

Mastico, R., Talbot, S. & Stockley, P. (1993). Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsids. *J. Gen. Virol.* **74**, 541-548.

Model, P. & Russel, M. (1991). Filamentous bacteriophages. In *The bacteriophages* (R. Calender ed.), vol. 2, pp. 375-456. Plenum Press, New York.

Morikawa, S., Booth, T. & Bishop, D. (1991). Analyses of the requirements for the synthesis of virus-like particles by feline immunodeficiency virus Gag using baculovirus vectors. *Virology* **183**, 288-297.

Nassal, M. & Schaller, H. (1993). Hepatitis B virus replication. *Trends Microbiol.* **1(6)**, 221-228.

Ni, C., White, C., Mitchell, R., Wickersham, J., Kodandapani, R., Peabody, D. & Ely, K. (1996). Crystal structure of the coat protein from the GA bacteriophage: Model of the unassembled dimer. *Protein Science* **5**, 2485-2493.

Nishihara, T., Harience, Y., Nozu, Y., Okada, Y. & Watanabe, I. (1969). Comparison of coat proteins from three groups of RNA phages. *Virology* **37**, 153-155.

Poggeler, S., Schwerk, C., Kamper, U. & Kuck, U. (1996). Efficient synthesis of a 72-kDa mitochondrial polypeptide using the yeast Ty expression system. *Biochem. Biophys. res. Commun.* **219**, 890-899.

Porta, C., Spall, D., Lin, T., Johnson, J. & Lomonossoff, G. (1996). The development of cowpea mosaic virus as a potential source of novel vaccines. *Intervirology* **39**, 79-84.

Priano, C., Arora, R., Butke, J. & Mills, D. (1995). A complete plasmid-based complementation system for RNA coliphage Qβ: three proteins of bacteriophages Qβ (group III) and SP (group IV) can be interchanged. *J. Mol. Biol.* **249(2)**, 238-297.

Pushko, P., Kozlovskaya, T., Sominskaya, I., Brede, A., Stankevica, E., Ose, V., Pumpens, P., & Grens, E. (1993). Analysis of RNA phage fr coat protein assembly by insertion, deletion and substitution mutagenesis. *Protein Eng.* **6**, 883-891.

Radloff, R. & Kaesberg, P. (1973). Electrophoretic and other properties of bacteriophage Qβ: the effect of a variable number of read-through proteins. *J. Virol.* **11(1)**, 116-128.

Rohrmann, G. & Kruger, R. (1970). The self-assembly of RNA free protein subunits from bacteriophage MS2. *Biochem. Biophys. Res. Commun.* **38(3)**, 406-413.

Schirmbeck, R., Bohm, W. & Reimann, J. (1996). Virus-like particles induce MHC class I-restricted T-cell responses. *Intervirology* **39**, 111-119.

Shouval, D., Ilan, Y., Adler, R., Deepen, R., Panet, A., Even-Chen, Z., Gorecki, M. & Gerlich, W. (1994). Improved immunogenicity in mice of a mammalian cell-derived recombinant hepatitis B vaccine containing pre-S1 and preS-2 antigens as compared with conventional yeast-derived vaccines. *Vaccine* **12(15)**, 1453-1459.

Smiley, B. & Minion, F. (1993). Enhanced readthrough of opal (UGA) stop codons and production of *Mycoplasma pneumoniae* P1 epitopes in *Escherichia coli*. *Gene* **134(1)**, 33-40.

Sominskaya, I., Pushko, P., Dreilina, D., Kozlovskaya, T. & Pumpens, P. (1992). Determination of the minimal length of preS1 epitope recignized by a monoclonal antibody which inhibits attachment of Hepatitis B virus to hepatocytes. *Med. Microbiol. Immunol.* **181**, 215-226.

Stahl, S., MacKay, P., Magazin, M., Bruce, S. & Murray, K. (1982). Hepatitis B virus core antigen: Synthesis in *E.coli* application in diagnosis. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1606-1610.

Takamatsu, H. & Iso, K. (1982). Chemical evidence for the capsomeric structure of phage Qβ. *Nature* **289(5877)**, 819-824.

Tars, K., Bundule, M., Fridborg K. & Liljas, L. (1997). The crystal structure of bacteriophage GA and a comparison of bacteriophages belonging to the major groups of *Escherichia coli* Leviviruses. *J. Mol. Biol.* **272**, 1-15.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76(9)**, 4350-4354.

Ulrich, R., Nassal, M., Meisel, H. & Kruger, D. (1998). Core particles of hepatitis B virus as carrier for foreign epitopes. *Adv. Viruss. Res.* **50**, 141-182.

Valegard, K., Liljas, L., Fridborg, K. & Unge, T. (1990). The three-dimensional structure of the bacterial virus MS2. *Nature* **345(6270)**, 36-41.

Valenzuela, P., Coit, D., Medina-Selby, M., Kuo, C., Van Nest, G., Burke, R., Bull, R., Urdea, M. & Graves, P. (1985). Antigen engineering in yeast: Synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1gD particles. *Bio/technology* **3**, 323-325.

Van den Worm, S., Stonehouse, N., Valegard, K., Murray, J., Walton, C., Fridborg, K., Stockley, P. & Liljas, L. (1998). Crystal structure of MS2 coat protein mutants

in complex with wild-type RNA operator fragments. *Nucleic Acids Res.* **26(5)**, 1345-1351.

Vaughan, T., Osbourn, J. & Tempest, P. (1998). Human antibodies by design. *Nat. Biotechnol.* **16(6)**, 535-539.

Wagner, R., Fliessbach, H., Modrow, S., von Brunn, A., Wolf, H., Boltz, T. & Gelderblom, H. (1991). Expression of HIV-1 autologous p55 and p55/gp120-V3 core particles: A new approach in HIV vaccine development. In *Vaccines 91* (Chanock, H., Ginsberg, F., Brown, F. & Lerner, R. eds.), pp. 109-114. Cold Spring Harbor Laboratory Press, New York.

Watanabe, I., Miyake, T., Sakurai, T., Shiba, T. & Ohno, T. (1967). Isolation and grouping of RNA phages. *Proc. japan. Acad.* **43**, 204.

Weber, K. & Koninsberg, W. (1975). Proteins of the RNA phages. In *RNA phages* (Zinder, N. ed.), pp. 51-84. Cold Spring Harbour Laboratory, USA.

Weiner, A. & Weber, K. (1973). A single UGA codon functions as a natural termination signal in the coliphage Qβ coat protein cistron. *J. Mol. Biol.* **80**, 837-855.

Zipper, P., Kratky, O., Herrmann, R. & Hohn, T. (1971). An x-ray small angle study of the bacteriophages fr and R17. *Eur. J. Biochem.* **18(1)**, 1-9.