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Chaperonin-Mediated Folding of Bacteriophage T4 Major Capsid Protein. II. Production of Gene Product 23 Deletion Mutants

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Abstract—Folding of bacteriophage T4 major capsid protein, gene product 23 (534 a.a.), is aided by two proteins: *E. coli* GroEL chaperonin and viral gp31 co-chaperonin. In the present work a set of mutants with extensive deletions inside gene *23* using controlled digestion with *Bal*31 nuclease has been constructed. Proteins with deletions were co-expressed from plasmid vectors with phage gp31 co-chaperonin. Deletions from 8 to 33 a.a. in the N-terminal region of the gp23 molecule covering the protein proteolytic cleavage site during capsid maturation have no influence on the mutants' ability to produce in *E. coli* cells proteins which form regular structures—polyheads. Deletions in other regions of the polypeptide chain (187-203 and 367-476 a.a.) disturb the correct folding and subsequent assembly of gp23 into polyheads.

Key words: bacteriophage T4, capsid, gene product 23, protein folding, co-chaperonin gp31

Bacteriophage T4, one of the basic objects of molecular biology over several decades, at present is widely used for study of protein folding mechanisms. Gene product 23 (gp23), the major structural capsid protein, is of particular interest. Gp23 folding is aided by the cooperation of two chaperonins: cell-host (*Escherichia coli*) GroEL and viral gp31 [1]. A characteristic of gp23 is its ability to assemble into different polymeric forms in the cell: during normal morphogenesis the protein forms procapsid and capsid, while in the presence of mutations in genes controlling procapsid core, gp23 forms polyheads.

Two steps through precursor-particle (procapsid) bring about phage capsid morphogenesis. Necessary for procapsid construction is an assistant temporal structure—protein nucleus or core, which mainly consists of gp22, and also a set of minor proteins (gp21, gp67, gp68, gpalt, IPI, IPII, IPIII) [2]. This core acts as a scaffold for polymerization of 960 copies of gp23 (534 amino acid residues (a.a.), 56 kD) [3], in which protomers arranged in hexamers form the procapsid shell having the shape of a prolate icosahedron. In the second step the prohead undergoes maturation when the virus-specific proteinase (gp21) proteolytically processes structural proteins. At the same time, specific cleavage of 65 amino acids from N-terminal part of gp23 molecule occurs, which is accompanied by profound rearrangements at the molecular level and results in translocation of at least two epitopes from inside the capsid surface to outside. When the procapsid molecular weight decreases by 20% the capsid volume expands by 50% due to lattice extension [4]. Structural reorganization is accompanied by DNA packaging into the capsid.

In chaperonin-mediated folding of gp23 the role of co-chaperonin is played by virus protein gp31 (111 a.a., 12 kD) that, like GroES, forms a stable complex with GroEL chaperonin in the presence of Mg-ATP and inhibits ATPase activity of GroEL *in vitro* [5]. According to X-ray structural data, in spite of weak homology of amino acid sequences, gp31 and GroES have in principle the same model of structure [6]. Active gp31 is also a heptamer and has toroidal shape, however the hole diameter in the gp31 heptamer is larger (2.6 nm), than in GroES (1.6 nm). Amino acid residues forming the toroidal hole are predominantly hydrophobic in GroES and hydrophilic in gp31 that is, probably, essential for gp23 folding. The more extended highly mobile loop of gp31, as compared to GroES, results in expansion of the cavity volume in the GroEL–gp31 chaperonin complex that is crucial for folding of the gp23 polypeptide chain, which, probably cannot be accommodated within the

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smaller confines of the cavity in the GroEL–GroES complex.

At present spatial structures of individual proteins (GroEL, GroES, and gp31) have been resolved [6-8], and the structure of relative complex GroEL–GroES has been determined [9]; however, structure and function of GroEL–gp31 complex in gp23 folding has not yet been ascertained.

Recently a plasmid vector for co-expression of gp31 and gp23 [10] allowing production the full-length gp23 has been constructed in our laboratory. In this system folding of recombinant gp23 occurs properly and protein assembles in *E. coli* cell into extended polymeric structures—polyheads. In the absence of gp31 the recombinant gp23 aggregates in the cell into amorphous lumps.

In the present work, to study major capsid protein folding and oligomerization, we have constructed a set of gp23 mutants with internal deletions in different regions of the polypeptide chain. Folding and subsequent oligomerization of N-terminal mutants with the help of GroEL–gp31 complex occurs correctly and results in formation of regular polymeric structures. The regions of polypeptide chain (187-203 and 367-476 a.a.), the deletions in which disturb gp23 folding and subsequent oligomerization, have been determined. As shown by homologous recombination *in vivo*, the deletions at the 5′-end of gene region encoded 60-88 a.a. of gp23 stop bacteriophage morphogenesis.

MATERIALS AND METHODS

Bacterial strains. Bacterial strain *E. coli* Top10 from Invitrogene (USA) was used for production of plasmid DNA and transformation while cloning. Strain *E. coli* BL21(DE3) from Novagen (USA) was used for expression of genes cloned into plasmids under control of the phage T7 promoter. Strains *E. coli* B178 (Su^-, non-permissive for amber mutants) and CR63 (Su^+, permissive for amber mutants) were used for titer determination of infective particles in homologous recombination test.

Design of deletion mutants. Deletion mutants were designed on the basis of expression vector pPK-31-23. Deletions in gene *23* were removed by controlled digestion with nuclease *Bal*31 [11]. Plasmid DNA was restricted at one of a unique restriction site (*Bst*EII, *Hind*III, *Hpa*I, *Nde*I) and linear DNA with protruding ends was used as a substrate for subsequent digestion with *Bal*31. Incubation was carried out at 25°C in buffer for *Bal*31. Aliquots (20 μl) were withdrawn from reaction mixture (100 μl) at 1 min intervals. The reaction was stopped by addition of 10 μl mixture of 0.1 M EGTA and 0.25 M EDTA. DNA 5′-ends were repaired with Klenow's fragment DNA polymerase I and ligated. Deletion length was determined by DNA sequencing.

Gene expression in *E. coli* BL21(DE3) cells. The genes cloned under phage T7 promoter were expressed as described earlier [10]. Competent BL21(DE3) cells were transformed with plasmid, plated onto dishes with LB-agar containing 1% glucose and ampicillin (100 μg/ml), and then were incubated for 12-18 h at 37°C. The colonies of transformants were inoculated in 5 ml of 2× TY medium containing ampicillin (200 μg/ml) and were grown at 37°C to optical density A_{600} ~ 1 (aliquots of transformants were stored with 10% glycerol at −70°C). To induce expression, isopropyl-1-thio-β-D-galactoside (IPTG) was added to the final concentration of 1 mM, and incubation was continued overnight at 25°C. The cells were centrifuged at 4000 rpm for 10 min using a Heraeus (Germany) Megafuge 2.0 R centrifuge.

Protein solubility analysis. Cell pellet was suspended in 100 mM potassium phosphate buffer, pH 7, and sonicated for 2-3 min (15 sec pulses with 15 sec pauses) on a UD-20 disintegrator (Techpan, Poland). Cellular debris was removed by centrifugation at 8800*g* for 10 min. Supernatant and pellet were analyzed by SDS-PAGE.

Electrophoretic analysis of recombinant proteins. A 100-μl sample was mixed with 30 μl of 4× sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, and 0.02% (w/v) Bromophenol Blue), boiled for 3 min, and 3-10 μl portions loaded onto the gel. Electrophoresis under denaturing conditions was carried out in 10 or 15% polyacrylamide gel according to Laemmli [12]. The gels were stained with 0.3% Coomassie R-250 from Sigma (USA) in acetic acid–ethanol–water (1 : 3 : 6 v/v) mixture and then distained with solution containing 50% ethanol and 7% acetic acid. A standard set of LMW proteins from Pharmacia and Biotech (Sweden) containing α-lactalbumin (14.4 kD), soybean trypsin inhibitor (20.1 kD), carboanhydrase (30 kD), ovalbumin (43 kD), BSA (67 kD), and phosphorylase *b* (94 kD) was used as the molecular weight marker.

Polymerase chain reaction. The multi-channel DNA amplifier MC2 (Russia) was used to perform the polymerase chain reaction (PCR). The reaction mixture (100 μl) for the standard amplification contained ~1 ng of DNA template, 50 pmol of each primer, 10 μl of 10× buffer from Promega (USA), 200 μM of each dNTP, and 2-5 units of Taq polymerase from Promega. Mineral oil (25 μl) was overlaid on the mixture. In all cases, the reaction was started by preliminary DNA denaturation at 95°C for 3 min; then 30 amplification cycles were carried out: 94°C (1 min), 53°C (1 min), 72°C (1.5 min). The products of amplification were analyzed in 1% agarose gel by their DNA mobility. Synthetic oligonucleotides 5′-AAG Gag AAt tCA AAT GAC TAT CAA A and 5′-AGG GAA CtC GAg GGT TCC C were used as forward and reverse primers, respectively.

Precipitation of polyheads by centrifugation. The pellet of cells after expression was suspended in 100 mM

potassium phosphate buffer, pH 7, and lysed with 0.5% chloroform (v/v) in the presence of 1 mM $MgSO_4$ and DNase I (0.05 mg/ml). Cellular debris was removed by centrifugation at 8800*g*. The supernatant was centrifuged at 30,000*g* for 1 h. The precipitated polyheads were resuspended in the initial volume of 100 mM potassium phosphate buffer, pH 7. The fractions were analyzed by electrophoresis.

Purification of polyheads. To purify polyheads, the supernatant obtained as described above was overlaid on 60% sucrose and centrifuged at 30,000*g* for 1 h. While *E. coli* proteins and gp31 remained in the supernatant, polyheads were concentrated on the sucrose "blanket". The supernatant was removed, and polyheads were dialyzed against 100 mM potassium phosphate buffer, pH 7, overnight.

Electron microscopy. The samples on the grids were contrasted with 0.75% uranyl formate. Microphotographs were obtained using a Hitachi 7000 electron microscope (Japan).

Homologous recombination *in vivo*. Recombination was carried out according to [13] using *amber* mutant on gene *23*—T4 B17 (am-268)—and mutants B8, B22, and B56 obtained by us. CR63 *E. coli* cells (permissive for *amber* mutants) were transformed by plasmid contained gene *23* with deletion, plated onto dishes with LB-agar containing ampicillin (100 μg/ml) and incubated at 37°C overnight. The transformants were grown in 4 ml of 2× TY medium to a density of $2 \cdot 10^8$ cells/ml, and then infected with T4 *amber* mutant B17 (1 : 3). After 1 h incubation at 37°C, the titer of phage particles on permissive strain CR63 and non-permissive B178 was determined.

RESULTS AND DISCUSSION

In this work we have constructed a set of plasmid vectors for expression of mutated gp23 with deletions inside the protein molecule. The mutants were designed on the basis of expression vector pPK-31-23 [10] that allowed producing in BL(21)DE3 *E. coli* cells bulk of soluble full-length gp23 assembled into polyheads. Gene *23* fragments ranging from 20 to 100 base pairs (bp) in length were removed by digestion with nuclease *Bal*31 after previous linearization of the original plasmid with respective restrictase at a unique restriction site. As a result, a set of deletions in gene *23* localized in restriction sites of *Bst*EII (I group mutants), *Hind*III (II group mutants), *Hpa*I and *Nde*I (III group mutants) was obtained. The constructions with high level expression of deletion forms of gp23 were selected by screening and solubility of recombinant proteins was analyzed.

Group I mutants (deletions of 49-89 a.a.). In gene *23* the deletions are localized in the *Bst*EII restriction site of the 5′-end (Fig. 1a). We have selected by screening 15 clones with high level expression of deletion mutants (Fig. 2a). The electrophoretic mobility of recombinant proteins in gel was no different from the mobility of full-length protein due to small length of deletions. The solubility test showed that only mutant (B9) produced insoluble protein, while the other mutants produced soluble proteins. According to gene *23* DNA sequencing data, the length of the removed protein fragments varies from 8 to 33 a.a. and the deletion limits correspond to Glu49 and Thr89 (table). The scheme of deletion location on gp23 primary structure and sequences of mutants in deletion region are represented in Fig. 1 (b and c). Additional centrifugation (15,000*g*, 15 min) of soluble fraction from cell lysate after expression results in quantitative precipitation of gp23, which is evidence of protein assembly into high-molecular-weight structures. It was supported by electron microscopy that these mutants, like native protein, actually form polyheads in *E. coli* cells. Electron microphotographs of polyheads formed by B67 (a) and B82 (b) mutants are shown in Fig. 3.

It should be noted that the given part of the gp23 polypeptide chain contains phage proteinase recognition site. As a result of the activation of this proteinase the cleavage of 65 a.a. fragment from the N-terminal of the molecule occurs during the maturation of the capsid. It was determined that proteolytic cleavage of full-length protein in this point *in vivo* is accompanied by lattice expansion of polymeric structures formed [3]. Possibly, polyheads assembled by deletion mutants also have different structure as compared with the native protein.

Proper folding and subsequent polymerization of monomers to polyheads are probably dependent on location of deletion in the mutants. Indeed, the deletion shift to N-terminal of the molecule results in folding failure: mutant B9 with deletion of 33 a.a. between Glu49 and Ala81 produces insoluble protein, while mutant B2 with the same length of deletion, but between Glu53 and Ser85 produces soluble product (table). Upon deletion of 25 a.a. from Asp52 (mutant B15) only about 50% of recombinant protein folds correctly and assembles into polyheads. Thus, deletion of polypeptide chain fragments of different length between Asp53 and Thr89 of the gp23 molecule N-terminal has no influence on proper folding and subsequent specific protein association into polyheads with the help of the chaperonin GroEL–gp31 complex.

The chaperonin complex carries out an important role in folding and polymerization of major capsid protein. It was determined that mutations in gene *31* result in capsid assembly prohibition, and at the same time gp23 amorphous aggregates form on the *E. coli* cytoplasmic membrane [14]. However, multiple point mutations in gene *23* have been known, when major capsid protein assembles into polymeric structures without assistance of chaperonin complex [15]. Study of folding and subsequent oligomerization in the absence of gp31 for some of the gp23 mutants obtained in this work is the next stage of investigation. Plasmid vectors lacking gene *31* have been

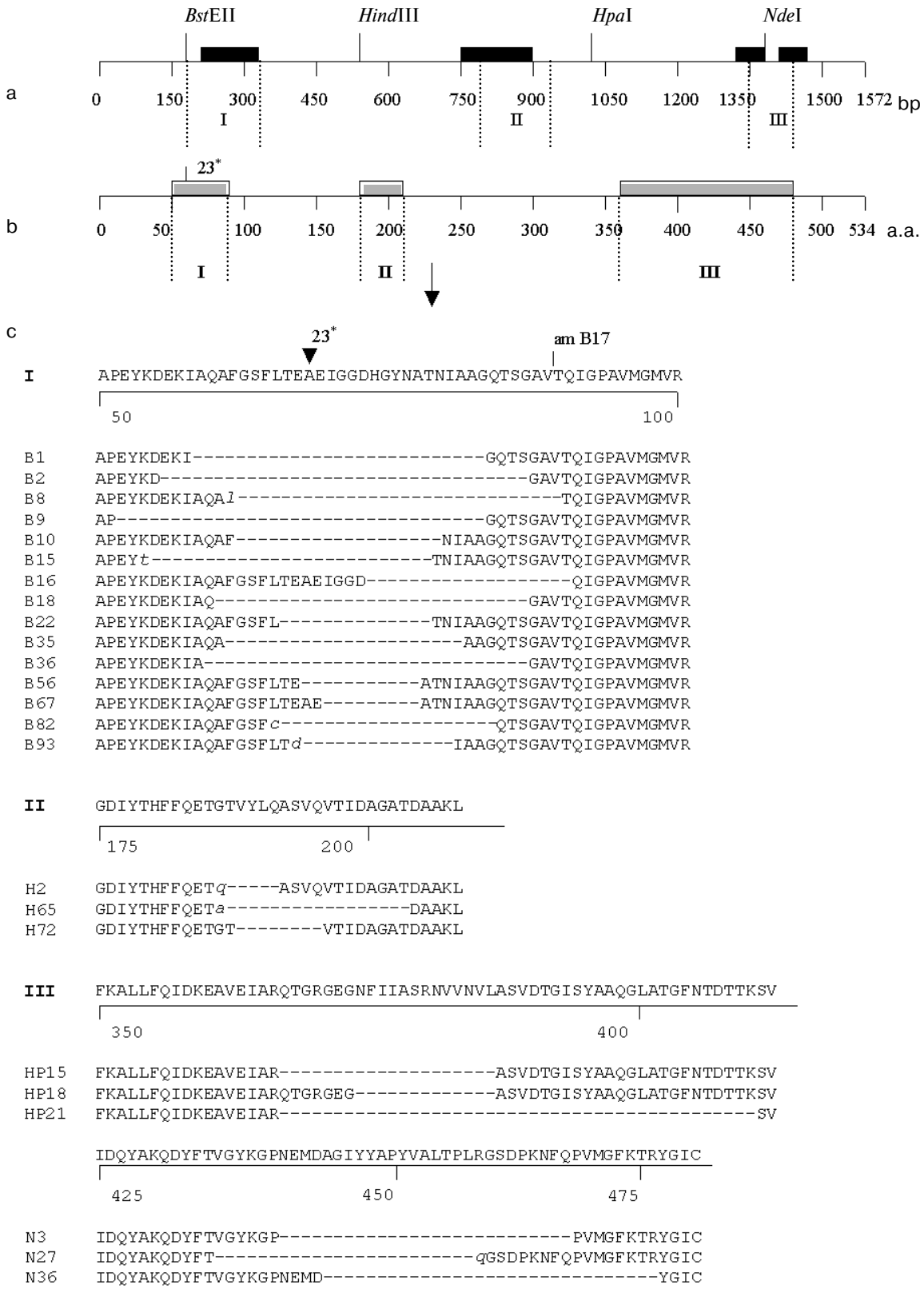

Fig. 1. a) Location of unique restriction sites in gene *23* and sequence homology to gene *24*. b) Location of deletions in mutants on gp23 polypeptide chain. Protein proteolytic cleavage site is 23*. c) Primary structure of gp23 mutants in deletion region. Dotted line, deleted fragments; italic, amino acid substitutions.

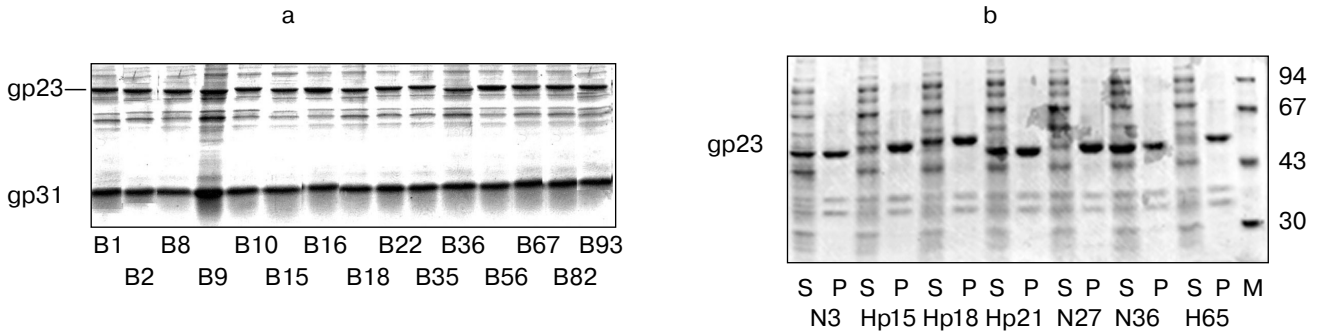

Fig. 2. Extracts of *E. coli* cells expressing gp31 and different deletion mutants of gp23 after induction with IPTG. Samples were analyzed in 15% (a) and 10% (b) SDS-polyacrylamide gel. S, supernatant; P, pellet; M, protein markers; to the right, their molecular weights in kD.

Deletion mutants of gp23

Mutant	Deletion limits	Deletion length, a.a.	Solubility (expression at 25°C)	Assembly of polyheads
B1	A56–A81	26	+	+
B2	E53–S85	33	+	+
B8	G60–V88	29	+	+
B9	E49–A81	33	—	—
B10	G60–N77	18	+	+
B15	D52– A76	25	±	—
B16	H72–T89	18	+	+
B18	A58–S85	29	+	+
B22	T64–A76	13	+	+
B35	I68–I79	12	+	+
B36	F59–S85	27	+	+
B56	A66–N75	10	+	+
B67	I68–N75	8	+	+
B82	T64–G82	19	+	+
B93	A66–N78	13	+	+
H2	T187–Q191	5	—	—
H65	T187–T203	17	—	—
H72	V188–Q195	8	—	—
Hp15	Q367–L386	20	±	—
Hp18	N374–V385	12	±	—
Hp21	Q367–K410	44	±	+
N3	N442–Q468	27	±	—
N27	V436–L459	24	—	—
N36	A446–R476	30	+	—

Note: ±, ≥50%; +, 90-100%.

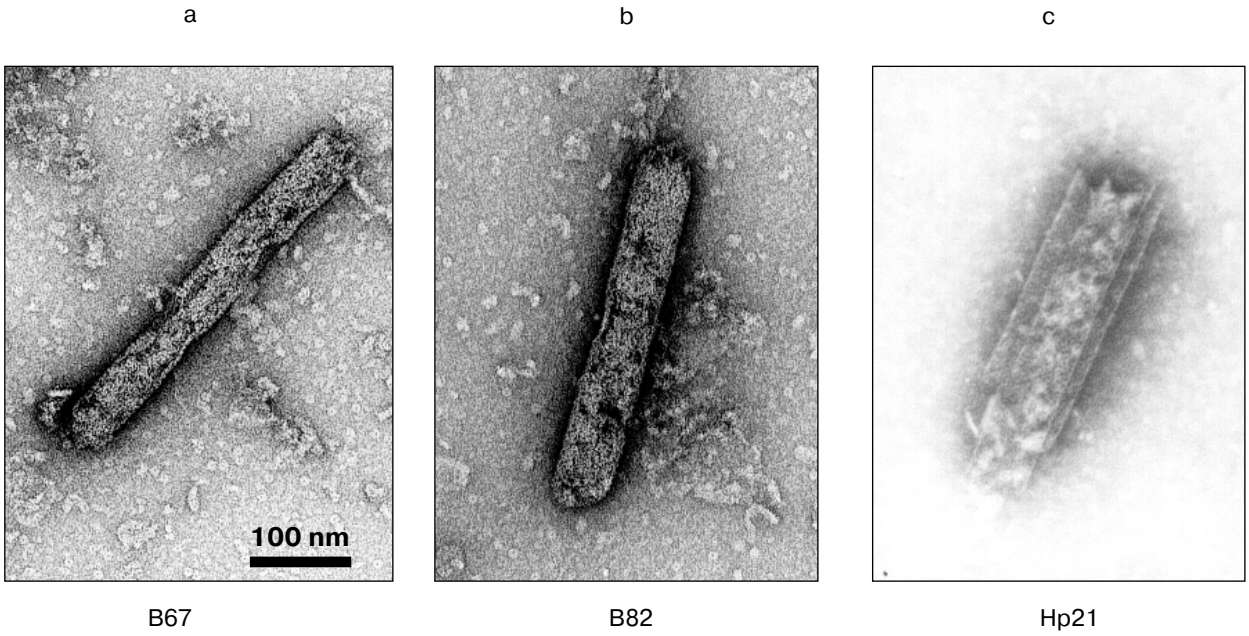

Fig. 3. Electron microphotographs of polyheads formed by mutants B67 (a), B82 (b), and Hp21 (c).

designed and expression of mutated gp23 in the absence of recombinant gp31 has been carried out. The DNA fragments contained different length deletions in gene *23* (mutants B8, B10, B22, B35, B56, B82) have been cut from plasmid vectors containing gene *31* and repeatedly cloned into pET-23d(+) vector by *Eco*RI-*Xho*I sites. The constructions were characterized by high level expression of gp23 (data not represented). However, in the absence of gp31 recombinant gp23 was produced by *E. coli* cell as inclusion bodies, which is evidence of protein folding and assembly failure.

Group II mutants (deletions of 187-203 a.a.). In gene *23* the deletions are localized in the *Hind*III restriction site (Fig. 1a). We have selected by screening six constructions (in the table three are presented). All constructions are characterized by high level expression of deletion gp23. Though the deletion length of this mutant group is not exceeding 17 a.a., all recombinant proteins are insoluble. Deletion of even short polypeptide chain fragments in this region of the molecule disturbs correct folding of the protein with the assistance of chaperonin complex.

Group III mutants (deletions of 367-476 a.a.). In gene *23* the deletions are localized in *Hpa*I and *Nde*I restriction sites (Fig. 1a). Six constructions produced mutated gp23 were selected by screening (table). The mutants Hp15, Hp18, and Hp21 contain gene *23* deletions in the region of the *Hpa*I restriction site, and deletion fragment length of gp23 polypeptide chain is 20, 12, and 44 a.a., respectively. Mutants N3, N27, and N36 have gene *23* deletions in the region of the *Nde*I restriction site, and the deletion length in the protein molecule is 27, 24, and 30 a.a., respectively. The deletions in this gene region result in expression at 25°C of partially soluble (Hp14, Hp15, Hp21, N3), soluble (N36) and insoluble (N27) proteins, while expression at 37°C results in production of only insoluble products. The electrophoretic picture of proteins distribution between supernatant and pellet fraction of cell lysate produced gp31 and gp23 mutants at 25°C is represented in Fig. 2b. The partially soluble mutants keep ability for specific association, but at that do not form pronounced polymeric structures. Among the mutants obtained only one (Hp21) forms polyheads, like full-length gp23 (Fig. 3c). Probably the deletion of Gln367–Lys410 is inessential for proper folding and assembly of major capsid protein. Upon deletion shift to the C-terminal of the molecule (mutant N36) the protein is produced in soluble form but not precipitated by centrifuging as in the case of native polyheads. Probably, as a result of folding disturbance, gp23 interaction regions become inaccessible to each other, so the protein loses completely its ability for specific association into regular structures. The deletion of a small polypeptide chain fragment in the region of Val436–Leu459 results in considerable conformational changes, as a consequence the mutant N27 produces insoluble protein.

The wide spectrum of properties of deletion mutants in the region of 367-476 a.a. is probably dependent on different protein conformational changes. Extended deletions apparently result in changes in gp23 structure; in that a part of the protein is produced in soluble form, but

it has lost its ability for specific association into polymeric structures. Deletions in this region possibly affect polypeptide chain fragments crucial for gp23 polymerization.

As known, gene *23* has homology regions to gene *24* (Fig. 1a). Point mutations in gene *23* resulted in formation of phage heads of different phenotype (giant or petite), and also temperature sensitive mutations form clusters (in Fig. 1a marked with dotted line) inside the sequence homology regions to gene *24* display [16]. It should be noted that deletion regions not affecting folding and polymerization of the major capsid protein are identical to location of gene *23* point mutations clusters and homology regions to gene *24* (Fig. 1, a and b). This coincidence is probably not accidental because not only deletion length effects on gp23 folding and oligomerization, but also its location on the polypeptide chain are seen.

Homologous recombination *in vivo*. Constructions B8, B22, and B56 designed by us have been used for substituting mutated gene *23* into the phage T4 genome by homologous recombination *in vivo* [13]. CR63 *E. coli* cells transformed by plasmid containing gene *23* with deletion were grown to a density of $2 \cdot 10^8$ cells/ml, infected with T4 *amber* mutant B17, and incubated at 37°C for 1 h. Then the titer of phage particles was determined on permissive (CR63) and restrictive (B178) strains. The titer of phage particles on CR63 and B178 was $2 \cdot 10^{10}$ and $2 \cdot 10^7$, respectively. Phage particles with integrated genome were selected on B178 strain. Gene *23* DNA from several plaques was amplified by PCR and its stability to *Bst*EII restrictase was analyzed. In all plaques, the DNA amplified contained a *Bst*EII restriction site that is evidence of the full-length gene *23* being presence. DNA sequencing results also confirmed the absence of deletion in gene *23*. Formation of viable phage particles in nonpermissive conditions is a result of homologous recombination, because the frequency of integration is 10^{-6}. Apparently, deletions at the 5′-end of gene *23* encoded N-terminal fragment from 60 to 88 a.a. of molecule stop bacteriophage morphogenesis.

The results of our study show a gp23 region (53-89 a.a.) where deletions do not affect proper folding and subsequent assembly of gp23 with assistance of GroEL–gp31 complex. Using these data we plan to select bypass mutants that are able to fold and assemble independently of chaperonin assistance.

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