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Bacteriophage T4 as a Surface Display Vector

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Abstract. We describe a method for construction of hymeric bacteriophage T4 particles displaying foreign polypeptides on their surface. The method is based on our finding that minor T4 fibrous protein fibritin encoded by gene *wac* (whisker's antigen control) could be lengthened at the C terminus without impairing its folding or binding to the phage particle. The lengthened fibritin gene could easily be transferred into the T4 genome by homologous recombination with a plasmid containing the modified gene *wac*. The modified gene *wac* is expressed properly during phage reproduction, and the lengthened fibritin is bound to phage particles. As an example of this type of method, we have obtained the hymeric T4 particles carrying a polypeptide of 53 residues, 45 of which are from the pre-S2 region of hepatitis B virus. The T4 display vector extends currently available display systems.

Key words: bacteriophage T4, fibritin, surface display vector, homologous recombination, HBV

The idea of using bacteriophages as a simple sensitive assay has a long history. Modified viable phages with small molecules attached to their surface by chemical procedures can be inactivated by antibodies against attached molecules that simplify the assay of specific antibodies or, in competition experiments, of attached molecules. This technique was first demonstrated in 1966 by Haimovich and Sela (1) and Mäkelä (2), who conjugated small haptenic groups to bacteriophages T4 and T2. Subsequently various haptenic groups, including biotin, have been attached to T4 particles (3, and references herein). Also, methods were developed for the covalent attachment of proteins to bacteriophage, resulting in viable preparations that were inactivated by antibodies against the antigenic determinants of protein (4). In 1982, Dulbecco (5) suggested that immunogenic peptides could be fused to the coat proteins of phage λ or other viruses. The resulting viral particles with new antigenic and immunogenic properties could be used as vaccines.

In 1985, Smith (6) demonstrated that the genome of filamentous *E. coli* phage *fd* could easily be manipulated to obtain phage particles displaying foreign polypeptides on their surface. In the following years surface display vectors were developed based on filamentous bacteriophages. They found numerous applications in protein engineering, antibody design, and epitope discovery (7–9) and were also shown to be promising models in vaccine development (10,11). In these vectors the protein or peptide incorporates into a phage particle via fusion with a structural protein, while the corresponding gene is packaged into the phage as part of the phage genome or phagemid. The physical linkage between the recombinant protein and the instruction for its production allows the display vectors to be selected by affinity purification followed by amplification via the normal phage growth cycle (12), which

provides a method for recovering polypeptides with required binding specificity from large combinatorial libraries. Similar display systems include peptide display on the surface proteins of *E. coli* (13), the "peptides-on-plasmids" approach (14), and, as reported recently, phage λ (15).

We describe a method for the display of recombinant proteins on phage T4 particles using the structural protein fibritin. Fibritin, encoded by the T4 late gene *wac* (whisker's antigen control), is a fibrous protein that builds the collar/whiskers complex on the phage neck (16). Although not essential for phage growth, the collar/whiskers complex has a helper function for long-tail fiber assembly (17,18). The *wac* gene encodes a polypeptide of 487 amino acid residues (19). We have shown that fibritin is a parallel triple-stranded α-helical coiled-coil rod that binds to the phage neck through the N-terminal domain, while the C-terminal domain is exposed outwards and is required for correct trimerization in vivo (20).

Taking into account the above-mentioned structure of fibritin in the whisker/collar complex, fusion of foreign polypeptides to the C terminus of fibritin seems to be a promising method for tethering polypeptides to the T4 neck. As deletions of the C-terminal residues have been found to be detrimental to the folding of fibritin (20), we tried lengthening the protein at the C terminus, leaving all of its residues intact. First, the TAA termination codon of the gene *wac* was changed into a TGC cysteine codon by site-directed mutagenesis, leading to the generation of *Sph*I and *Nco*I restriction sites (Fig. 1). Using the methology described previously (20), the recombinant protein with 15 additional residues encoded by the short untranslated region between genes *wac* and 13 (21) was found to fold correctly and to be biologically active. Then, the DNA fragment of hepatitis B virus (HBV) (subtype *ayw*) encoding for residues 112–156 of the pre-S2 region (22) was inserted at the end of the modified *wac* gene (Fig. 1). The resulting changes in the T4 DNA sequence can be viewed as an insertion of a 159 bp DNA fragment immediately before the termination codon TAA of the gene *wac*. The lengthened fibritin carries an additional 53 residues at the C terminus, 45 residues of which are from HBV. The protein was expressed from the T7 promoter of the vector in large amounts in a soluble form and was purified by the procedure we described fornative and truncated fibritins (20) (Fig. 2A, lane 1).

In the pRR2 plasmid the gene *wac* is flanked

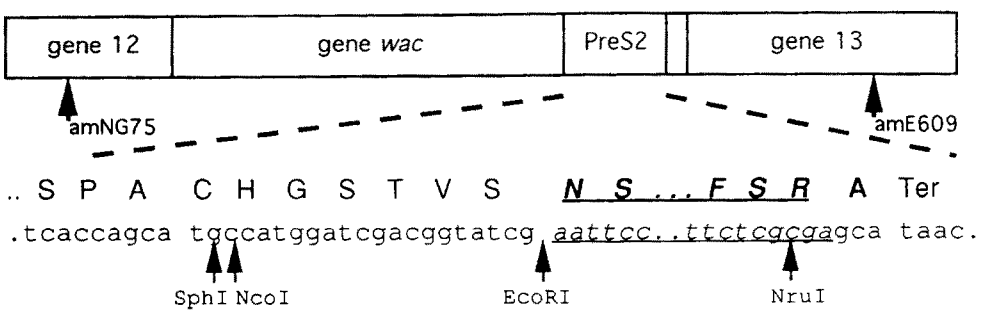

Fig. 1. Structure of the insert in the pRR2 plasmid. Nucleotide and amino acid residues exogenous to T4 phage are shown in bold italic letters. The protein sequence from HBV (residues 112–156 of pre-S2 region) (22) and the DNA encoding it are underlined. Amber mutations used in this study are indicated. The plasmid is a pTZ19R vector (Pharmacia) carrying the *Xba*I-*Eco*R47III restriction fragment of phage T4 DNA containing the lengthened gene *wac* and parts of genes 12 and 13 (21) cloned at the *Hind*III-*Eco*RI sites of the vector polylinker. To allow cloning, *Eco*RI ends have been completely blunted, while *Xba*I and *Hind*III were blunted partially with the Klenow fragment of *E. coli* polymerase. Modification of the end of the gene *wac*, detailed in the insert, was conducted in several steps with multiple subclonings. A detailed description is given in Efimov (26). The blunted *Eco*RI-*Taq*I fragment of the HBV genome, spanning nucleotides 3055–3182, 1-4 (22), has been used. The *Nru*I site at the end of the HBV sequence occurred as a result of our subcloning. The *Sph*I, *Nco*I, and *Nru*I sites at the end of the gene *wac* were constructed by site-directed mutagenesis. The short region between the *Nco*I and *Eco*RI sites originates from vectors used in subcloning. The bacteriophage T4 genes 12, *wac*, and 13 are deposited in the EMBL Gene Data Bank under accession numbers X56555, 12888, and X14868, respectively.

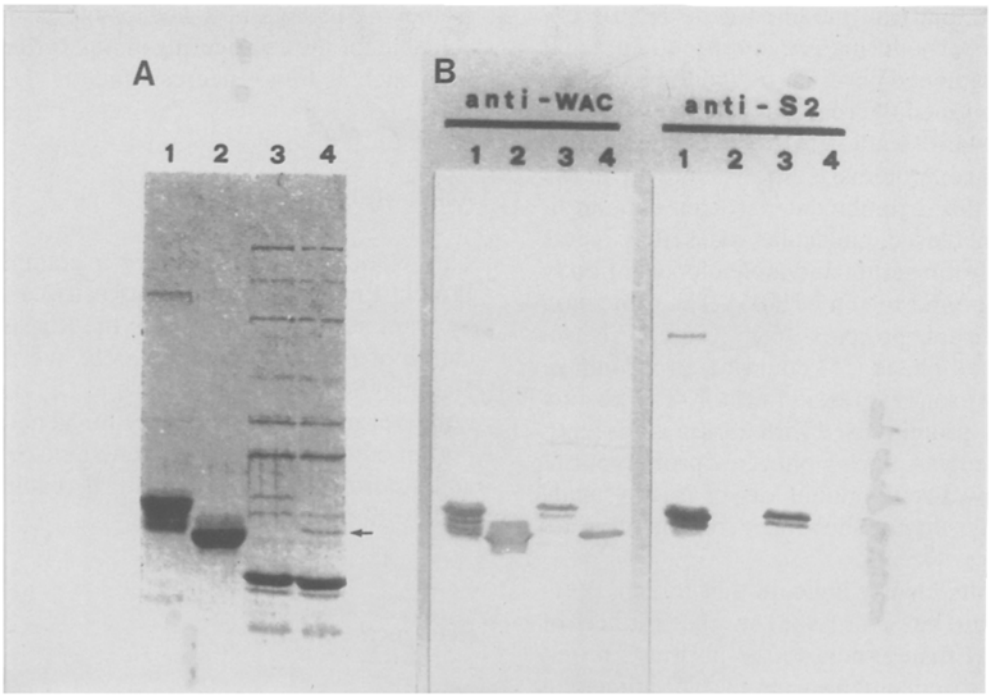

Fig. 2. Analysis of recombinant T4 particle proteins. Recombinant and wild-type T4 particles were grown on *E. coli* B/1 cells and were purified by sedimentation through a sucrose gradient (27). Purified proteins were prepared by the expression of the plasmids pWAC (20) and pRR2 as described earlier (20). Samples were analyzed by 8% $NaDodSO_4$-PAGE and either stained with Coomassie (*A*; arrow shows the position of gene product *wac*) or probed by Western blot (*B*) with anti-fibritin rabbit serum and monoclonal antibody against the pre-S2 epitope of HBV (S 2.5, Immunotech SA). Lane 1, purified lengthened fibritin; lane 2, purified native fibritin; lane 3, recombinant T4 phage; lane 4, T4 wild type.

by fragments of genes 12 and 13. In each of these genes an amber mutation, designated as NG75 and E609, respectively, has been mapped (23 and our data). These mutations were used as markers for the transfer of the modified *wac* gene into the T4 genome by in vivo recombination with the pRR2 plasmid according to the procedure of Volker et al. (24). *E. coli* CR63 cells, which are permissive for amber mutants, were transformed by the pRR2 plasmid. The cells were grown to the exponential phase (2×10^8 cells/ml) and then were infected by the T4 NG75-E609 double amber mutant. To reveal the recombinant phages carrying no amber mutations, the progeny was plated on *E. coli* B/1 cells nonpermissive for amber mutations. After recombination, the titer of wild-type phage progeny rose to $1–2 \times 10^6$ plaque forming units (pfu)/ml. In control experiments employing recombination with empty vector or vector carrying only genes 12 or 13, the titer on B/1 was less than 10^2 pfu/ml. The titer on CR63 cells was around $1–2 \times 10^{10}$ pfu/ml.

The lysates of eight independent recombinants were analyzed by $NaDodSO_4$-PAGE and immunoblotting with rabbit anti-fibritin serum. In six cases a single band of 58 kD molecular weight corresponding to the lengthened fibritin was revealed. The other two recombinants contained wild-type 52 kD fibritin. This was also confirmed by polymerase chain reaction (PCR) of the recombinant phage plaques using the primers complementary to the end of the gene *wac* and to the beginning of the gene 13. Thus, the efficiency of cloning in T4 phage using in vivo recombination with the double-amber mutant is about 75%. The plaque morphology of recombinant phages did not differ from the wild-type T4. However, when prepared by growing in liquid culture, their titers were usually two- to threefold lower than the wild type.

To prove that the modified gene *wac* is expressed properly during phage reproduction and that the lengthened fibritin is bound to phage particles, we purified the recombinant and wild-type phages by sucrose gradient centrifugation. Analysis of phage proteins is shown in Fig. 2. As predicted, the recombinant particles contain fibritin of increased molecular mass that reacts with anti-fibritin serum and monoclonal antibody specific to pre-S2 region of HBV. The protection of recombinant proteins from proteolysis reported for T4 phage (25) could be an advantage of using T4 as an expression vector. The portion of the HBV protein fused with fibritin is susceptible to proteolysis during phage or protein purification. However, immunoblots of freshly made phage lysates do not show the products of proteolysis.

Our results clearly indicate that foreign polypeptides can be expressed on the surface of phage T4 by fusing them to the fibritin C terminus with subsequent transfer of the chimerical gene into the T4 genome by homologous recombination with the plasmid. Like a recently devised λ phage vector (15), a vector based on phage T4 might be useful for the display of proteins that cannot be secreted. In some aspects, the structural framework of displayed polypeptides in our system is different from what was described previously. As they are outwardly exposed, the C termini of whiskers provide excellent access to the displayed molecules. As each whisker is composed of three chains and there are six of them on a phage particle, the avidity of displayed molecules to their ligands will increase. It is possible to obtain homogenic preparation of recombinant phages with all fibritin molecules substituted by lengthened molecules. On the basis of published data on inactivation of phage T4 by antibodies against fibritin (17,18), direct inactivation of recombinant T4 particles by ligands of displayed molecules is unlikely to be a very sensitive detection assay. Titering of chimerical phages after their binding to immobilized ligand could be a considerably more sensitive and quantitative method of detection. Like other display systems, the bacteriophage T4–based system could also have application in selection from protein and peptide libraries, and in mimicking of epitopes, and could be especially valuable for the engineering of homotrimeric proteins such as tumor necrosis factor.

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