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Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface

Abstract. *Foreign DNA fragments can be inserted into filamentous phage gene III to create a fusion protein with the foreign sequence in the middle. The fusion protein is incorporated into the virion, which retains infectivity and displays the foreign amino acids in immunologically accessible form. These "fusion phage" can be enriched more than 1000-fold over ordinary phage by affinity for antibody directed against the foreign sequence. Fusion phage may provide a simple way of cloning a gene when an antibody against the product of that gene is available.*

Gene III of filamentous phage encodes a minor coat protein, pIII, located at one end of the virion (*1, 2*). The amino-terminal half of pIII binds to the F pilus during infection, while the carboxyl-terminal half is buried in the virion and participates in morphogenesis (*3, 4*). I report here that a foreign sequence can be inserted between the two domains without unduly disrupting pIII function and that the foreign sequence is displayed in immunologically accessible form on the infectious particle. Affinity purification of such "fusion phage" may provide a way of cloning a gene from a library of random inserts when antibody against the gene product is available; this scheme could be simpler than present methods, in which antibody is used indirectly to screen a conventional expression-vector library for the desired clones (*5*).

As a source of model inserts I used a Sau 3A digest of plasmid pAN4, which encodes the Eco RI endonuclease and methylase enzymes (*6*); the gene III insertion site was the unique Bam HI site of phage f1 (*7*). Several pAN4 fragments were candidate inserts, in that they would preserve the gene III reading frame without stop codons when ligated into the Bam HI site; the other fragments would result in noninfective particles. Two candidate inserts derive from the endonuclease gene: a 132-base-pair (bp) fragment (nucleotides 416 to 547) and a 171-bp fragment (nucleotides 548 to 718) (*6*); a mutant pIII with these inserts would bear a segment of endonuclease sequence.

Accordingly, a Sau 3A digest of pAN4 was ligated with Bam HI–cleaved f1 DNA and transfected into *Escherichia coli* strain K38 (legend to Fig. 1) (*8*). Twenty-three of the 24 clones examined could not be cleaved by Bam HI and thus were presumed to harbor gene III inserts. By restriction analysis at least three different inserts could be distinguished, including the 171-bp (but not the 132-bp) endonuclease fragment.

One clone with the 171-bp insert, fECO1, was propagated and studied further. Antibody to Eco RI endonuclease (*9*) markedly reduced the fECO1 titer, but it had no effect on the f1 parent; the phage-neutralizing activity of the antibody could be completely blocked by preincubation with purified endonuclease (Fig. 1) (*10*). This demonstrates that antigenic determinants coded by foreign inserts can be displayed on the virion surface. Buffer at *p*H 2.2 or 2.7 reversed the reaction of phage with antibody, restoring most or all of the original titer.

Phage fECO1 gave very small plaques on strain K38 and almost invisible plaques on strain JM107 (*11*). The stationary phase titer was 100 times lower than normal; however, the number of physical particles (quantified spectrophotometrically) (*12*) exceeded the number of plaque-forming units (PFU) by a factor of more than 50, compared with a factor of about 2 for wild-type f1. These results indicate that the phage are partially defective in infectivity. They also show a considerably higher incidence of multimeric "polyphage" (*1*) than normal, as determined by whole-virion electrophoresis (*13*) and electron microscopy. Neither abnormality is surprising in view of pIII's role in infection and morphogenesis (*3, 4*). These defects probably put fusion phage at a selective disadvantage with respect to variants in which the insert has been lost or altered. Indeed, one attempt at propagating fECO1 produced phage with an apparently altered or deleted insert, as they could not be detectably neutralized with antibody to endonuclease. The accumulation of unwanted variants might be circumvented by using fd-tet as the vector, since it can be propagated as a plasmid, independently of gene III function (*4*).

In the following experiment I tested the ability of antibody to affinity-purify cognate fusion phage from a large background of noncognate phage, such as would be present in a fusion phage library. Antibody was adsorbed to a small polystyrene petri dish, the remaining adsorption sites being blocked with excess protein. A suspension containing fECO1 phage and a large excess of the double-amber mutant strain M13mp8 (*14*) was swirled in the dish, which was then washed extensively with fresh medium. The dish was eluted with acidic buffer, and the fECO1 and M13mp8 phage in the eluate were separately titered.

As shown in Table 1, fECO1 was enriched over M13mp8 by factors of 1500 to 7200 in the three experiments. Detailed analysis of the results of experiment 3 suggested two ways in which purification might be improved (Table 1). First, there were appreciable M13mp8 phage but few fECO1 phage in the last three washes, suggesting that further washing might be useful. Second, there were considerably more M13mp8 phage

Table 1. Affinity purification of fECO1 phage with Eco RI antibody. The immunoglobulin G fraction of rabbit antiserum to Eco RI endonuclease (*9*) was prepared by chromatography on DEAE–Affigel Blue (BioRad) and filter-sterilized. Six milliliters of antibody at 60 μg/ml in filter-sterilized 0.2*M* $NaHCO_3$ with HCl (*p*H 9.2) was swirled in a 60 by 20 mm polystyrene petri dish for 1 to 2 hours. Antibody was removed and stored in a sterile glass vial at −20°C for use in later experiments. Six milliliters of filter-sterilized blocking solution [2.9 percent (by weight) bovine serum albumin in 0.15*M* NaCl and 10 m*M* tris-HCl (*p*H 7.5)] was pipetted onto the dish, which was gently swirled for 20 to 45 minutes. The dish was rinsed with 6 ml of TY medium, and 6 ml of TY containing 9.2×10^6 PFU of fECO1 and 3.8×10^{11} PFU of M13mp8 (*14*) was pipetted into the dish, which was swirled for 3, 22, or 18 hours for experiments 1, 2, and 3, respectively. Phage were removed and the dish was washed five to ten times with 6 ml of TY medium. Six milliliters of elution buffer (0.1*N* HCl adjusted to *p*H 2.2 with glycine) with various concentrations of NaCl and gelatin was pipetted into the dish, which was swirled for 20 to 45 minutes. In experiment 1 the elution buffer contained no NaCl and 0.25 percent (weight to volume) gelatin; in experiment 2, 1*M* NaCl and 0.25 percent gelatin; and in experiment 3, 1*M* NaCl and 0.5 percent gelatin. The eluate was neutralized to *p*H 8 with 1 ml of 1*M* tris. The fECO1 phage were titered on log-phase K38 cells [a nonamber-suppressing strain that does not plate M13mp8 (*8*)]; M13mp8 phage were titered on log-phase JM107 cells (*11, 14*). N.D., not determined.

Fraction	Yield (% input)		Enrichment
	fECO1	M13mp8	
	Experiment 1		
Final eluate	0.46	1.4×10^{-4}	3.4×10^3
	Experiment 2		
Final eluate	0.88	1.2×10^{-4}	7.2×10^3
	Experiment 3		
Phage after binding	62.1	92.2	
Wash 1	0.41	N.D.	
Washes 2 to 7	0.020	N.D.	
Wash 8	0.003	6×10^{-6}	
Wash 9	0.001	7×10^{-6}	
Wash 10	0.004	1×10^{-5}	
Final eluate	0.31	2.0×10^{-4}	1.5×10^3

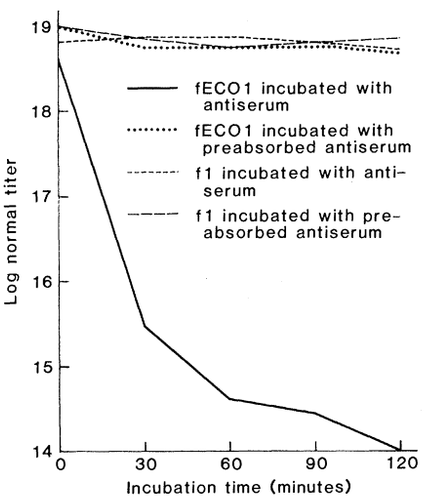

Fig. 1. Neutralization of fECO1 phage by endonuclease antibody. Phage, either fECO1 or wild-type f1, were incubated for various times with untreated antiserum or antiserum that had been preabsorbed with an excess of purified Eco RI endonuclease. Unless otherwise noted, phage were grown and titered as described by Salivar *et al.* (*15*), and DNA was manipulated as described by Maniatis *et al.* (*16*). Insert-bearing clones were constructed by ligating 250 ng of Sau 3A–cleaved pAN4 and 250 ng of Bam HI–cleaved f1 replicative form (RF) in 53 μl of 50 m*M* tris-HCl (*p*H 7.6), 10 m*M* $MgCl_2$, 4 m*M* dithiothreitol, 1 m*M* adenosine triphosphate, 5 U of T4 DNA ligase, and 10 U of Bam HI (Bethesda Research Laboratories). After 16 hours at 22°C and 90 minutes at 65°C, 2.7 μl of 1*M* NaCl and 10 U of Bam HI were added and the mixture was incubated for 2 hours at 37°C. Volumes corresponding to 160, 16, and 1.6 ng of f1 DNA were transfected into K38 cells (*13*), yielding 250, 104, and 17 plaques, respectively, most of which were smaller than normal. Twenty-four small plaques were inoculated into 1-ml early log-phase cultures of K38 cells in TY medium (10 g of Bacto Tryptone, 1 g of yeast extract, 8 g of NaCl, 0.2 g of $CaCl_2$, and 2 g of glucose, all dissolved in 1 liter of water and autoclaved) and shaken overnight at 37°C. Crude RF DNA from the cells was analyzed by restriction with Bam HI, Hind III, and (in some cases) Hph I. Clone fECO1 was plaque-purified and propagated in a 50-ml culture as described above. Virions were purified from the supernatant (*13*), and RF was prepared from the cells by base-acid lysis and chromatography on NACS-37 (Bethesda Research Laboratories). Acrylamide gel electrophoresis of Sau 3A and Hph I digests confirmed the structure of the fECO1 RF molecule. Phage neutralization was carried out as follows. Endonuclease antiserum (*9*) diluted 232.5-fold in TY medium; to 27.9-μl aliquots of this dilution was added either 2.1 μl containing 3.74 μg of purified endonuclease (*10*) or 2.1 μl of blank endonuclease buffer. Half the contents of each tube (15 μl) was pipetted into a second tube, and all tubes were incubated for 1 hour at 37°C. Then 15 μl of fECO1 or f1 phage diluted to a nominal titer of 3.0×10^8 PFU/ml in TY medium was added to the tubes and incubation was continued at 37°C. At various times 5-μl aliquots were removed and diluted 100 times in ice-cold TY medium. At the end of the incubation period all the 100-fold dilutions, and dilutions of those dilutions, were plated on log-phase K38 cells (*8*), yielding the titers shown.

in the eluate than in the last wash, showing that they were bound relatively tightly to the dish and suggesting that blocking the dish with noninterfering phage (such as double-amber mutants when both the cognate fusion phage and the background phage are not amber mutants) might increase the enrichment factor.

The success of the foregoing affinity-purification experiment gives hope that antibody might be used to isolate desired clones from a library of random inserts in a fusion-phage vector. Thousandfold enrichments can be expected after a single round of purification, and presumably much greater enrichments can be expected after a second round; purity could conveniently be monitored by phage neutralization. Fusion phage libraries would have to be constructed on a larger scale than libraries of conventional expression vectors in order to ensure representation of a given sequence, since a foreign coding sequence must be correctly fused to the gene III reading frame on both sides (not just one) to be expressed. Moreover, it is unlikely that fusion phage will accommodate inserts as large as the average coding sequence expressed in a conventional expression vector, necessitating a further increase in the scale of construction. On the other hand, most unwanted inserts—those from noncoding sequences and those in which a natural coding sequence is fused in the wrong reading frame—will cause premature termination of translation, and therefore will not contribute to the pool of infective phage from which desired clones must be enriched. In conventional vectors, by contrast, target clones must be enriched from a pool that includes a clone, regardless of whether it expresses a natural coding sequence.

Finally, filamentous phage are immunogenic, and immunization with fusion phage might elicit antibodies against the foreign determinant. This could be a useful way of obtaining antibodies against restricted determinants or of producing vaccines of medical or veterinary interest.

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Phosphate Release and Force Generation in Skeletal Muscle Fibers

Abstract. *Rapid laser pulse–induced photolysis of an adenosine triphosphate precursor in muscle fibers abruptly initiated cycling of the cross-bridges. The accompanying changes in tension and stiffness were related to elementary mechanochemical events of the energy-transducing mechanism. When inorganic phosphate was present at millimolar concentrations during liberation of adenosine triphosphate in the absence of calcium, relaxation was accelerated. Steady active tension in the presence of calcium was decreased but the approach to final tension was more rapid. These results suggest that, during energy transduction, formation of the dominant force-generating cross-bridge state is coupled to release of inorganic phosphate in a reaction that is readily reversible.*

Inorganic phosphate (P_i) is present in muscle cells at millimolar concentrations, and during activity or fatigue the P_i concentration increases severalfold (*1*). Inorganic phosphate reduces the steady tension (*2, 3*) and stiffness (*2*) of isometrically contracting skinned muscle fibers. We report here experiments involving a photochemical technique that abruptly initiates the actomyosin cross-bridge cycle in skinned fibers. The results suggest an important role for P_i release in the cross-bridge cycle.

Single skinned fibers from rabbit psoas muscle were equilibrated in the rigor state with 10 m*M* "caged" ATP [P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate] (*4*), a photolabile precursor of ATP. Tension and stiffness transients were monitored as ATP (500 to 800 μ*M*) was rapidly liberated from caged ATP in the fiber by a pulse of radiation (wavelength, 347 nm) from a frequency-doubled ruby laser (*5*). A piezoelectric element applied a 500-Hz, 1-μm sinusoidal length oscillation to the fiber for stiffness measurements and the tension was detected by a high-bandwidth transducer (*5, 6*). A phase-sensitive lock-in amplifier separated the sinusoidal portion of the transducer signal into components in phase and 90° out of phase with the change in length. The amplitudes of these sinusoidal components are qualitatively related to the mechanical state of the cross-bridges. If we assume that the elasticity of an attached cross-bridge is fixed and neglect artifacts due to end compliance, then the in-phase sinusoidal component is the stiffness proportional to the number of attachments (*5*). The 90° out-of-phase component (quadrature stiffness) indicates viscoelasticity of the attached cross-bridges. After a small abrupt change in length, during an active contraction, the tension recovers within a few milliseconds to almost its value before the change in length (*7*). This quick recovery is diminished or absent in rigor (*8*). With sinusoidal changes in length, the quick recovery of active cross-bridges results in a phase shift of the sinusoidal tension response (*9*). In our experiments a positive deflection of the quadrature stiffness traces resulted from this phase shift and thus indicated the viscoelasticity of cross-bridges actively producing force (*5, 10*).

Figure 1 (A and B) shows superimposed tension recordings from successive rigor-photolysis-relaxation trials in the absence of Ca^{2+}. In one trial the fiber was held isometric and in the other it was stretched 0.53 percent 1 second before photolysis. After the liberation of ATP there was a rapid drop in tension due to cross-bridge detachment. The two tension traces then converged and remained superimposed during the final relaxation.

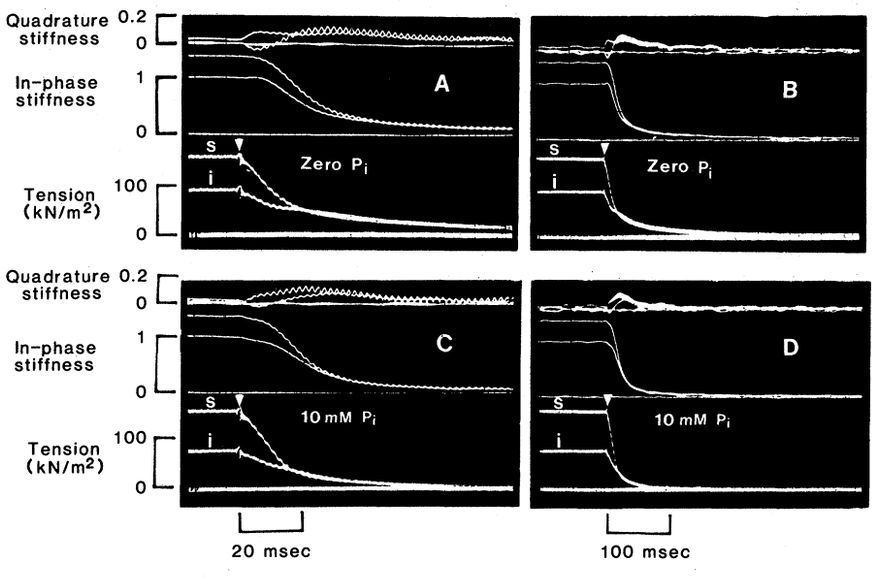

Fig. 1. (A to D) Tension and stiffness transients recorded during photolysis of caged ATP in a single glycerol-extracted fiber held isometric (i) or stretched (s). Shown are fast (A and C) and slow (B and D) time-base recordings obtained during trials without (A and B) and with (C and D) 10 m*M* orthophosphate (P_i). The fiber was equilibrated with a rigor-inducing solution containing 20 m*M* EGTA, 100 m*M* trimethylaminoethanesulfonic acid, 1 m*M* Mg^{2+}, $<10^{-8}$ *M* Ca^{2+}, 10 m*M* reduced glutathione, 0 to 10 m*M* P_i, 10 m*M* caged ATP, and 15 to 25 m*M* 1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (final ionic strength, 200 m*M*; *p*H 7.1; 22°C). The oscilloscope sweeps were triggered before photolysis, and the mechanical transients induced by the liberation of 700 μ*M* ATP (arrowhead) were recorded (*5, 6*). Stiffness was monitored at 500 Hz. Stiffness traces have been normalized to the in-phase rigor stiffness. Baseline stiffness and tension measurements are those of the fully relaxed fiber. The first 10 msec of the stiffness records contain artifacts associated with the laser pulse.