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Expression cloning of cDNA by phage display selection

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ABSTRACT

Expression cloning of a mouse kappa chain fragment has been achieved from a cDNA library by display of expressed proteins on filamentous phage and affinity selection for binding to anti-mouse Fab antibodies. Expressed proteins were anchored to the phage coat by a synthetic, anti-parallel leucine zipper, which had been selected from a semi-randomized zipper library for the ability to connect a test protein to phage. From a library of 4×10^6 transformants, two separate clones displaying different size cDNA inserts were recovered after four selection rounds. These results further demonstrate the utility of phage display for cDNA expression cloning.

Phage display has become a well demonstrated method for identification of peptide and protein ligands due to its speed and sensitivity (1–5). Bacteriophage constructed to display expressed foreign proteins from a DNA based library can be selected by the unique binding ability of the foreign surface protein to an immobilized target, thereby isolating the gene responsible for the displayed protein. Experimentally, this selection allows libraries of 10^8–10^9 to be accessed, with recovery of affinities as low as 10^5–10^6 M^{-1} (11), at least two orders of magnitude better than can be expected by filter lifts.

Libraries from DNA with well defined 5′ and 3′ gene sequences such as random peptides, mutants of a single protein, or antibodies are simple candidates for phage display, since it is relatively straightforward to insert genes for display between a periplasmic export leader sequence and N-terminus of the phage minor or major coat proteins gIIIp or gVIIIp, matching the two reading frames. On the other hand, cDNAs with indeterminate 5′ and 3′ ends and potential translational stops are not straightforward display candidates. Thus systems that use attachments other than fusion to N-termini of phage coat proteins have been developed for display of expressed cDNA (6,7). One system uses fusion to the C-terminus of another minor phage coat protein, gVIp, yet the display efficiency is 100 times less than a comparable gIIIp based system (6). Another example is the attachment of the displayed proteins by fusion to the C-terminus of a Fos leucine zipper that pairs with a complementary Jun zipper fused to gIIIp (7). This method was successfully used in cloning ligands to human IgE from an *Aspergillus fumigatis* cDNA library (8), although it used vectors known to have deletional instability (9).

We chose to adopt a strategy based on a leucine zipper to attach translated cDNA proteins to phage (7,8), but with three important considerations. First, different promoters and periplasmic export leader sequences were chosen to avoid deletional instability. Second, the amino acid sequence of the zippers needed to be chosen such that they were adequately translated and transported to the periplasm where they would associate. Third, the optimal orientation of the zippers, either parallel or antiparallel, would have been hard to predict since little structural detail about filamentous phage was known. Therefore, selection of zippers from a partially random library was undertaken. A zipper library was designed to contain both parallel and antiparallel zipper pairs in the following manner: the *a* and *d* positions of the heptad repeat were fixed to be leucine; the *e* and *g* positions were randomly glutamate or lysine; the *b*, *c* and *f* positions were amino acids commonly found in coiled coils (12); and, finally, the terminal *a* and *d* positions were chosen to be cysteine, to prevent scrambling of displayed proteins among different phage. The partially degenerate nucleotide sequence used to create the zipper library is shown in Figure 1A. Sequential insertion of this sequence into the zip1 and zip2 sites of the phagemid display vector (Fig. 1B) gave a zipper library with 8×10^6 primary transformants, of sufficient size to sample all possible combinations of glutamate and lysine in the *e* and *g* positions, 7×10^4, which are the primary determinants of zipper orientation (13).

Panning the hybrid phage library as previously described (10) for phage that display BAP (Fig. 1D, triangle) resulted in functional zippers, as demonstrated by test panning individual clones selected from the fourth round (Fig. 1C). Phage with zipper sequences in clone 66-12 or 66-8 can display BAP and an epitope tag and bind to wells coated with anti-BAP or anti-TAG antibodies at least 1000-fold better than they bind to BSA alone. Deletion of TAG and BAP sequences abolished affinity of phage bearing zippers to anti-TAG and anti-BAP antibodies (Fig. 1C). Comparing possible electrostatic interactions of the *e* and *g* side chains based upon rules developed for coiled coils (13), an antiparallel arrangement of the two zippers with six attractions and two repulsions would be more favored over parallel with four attractions and four repulsions (sequence zip1, CEKLEAK LKELERK LAQLKRE LKKLEAK LEEC; zip2, CATLKGE LTALRAE LVQLERE LTALKGE LEEC). The proportion of phage displaying BAP fusion protein was measured by a functional panning assay. Ten-fold serial dilutions of recombinant phage clone 66-12 were subjected to panning against the anti-BAP antibody, and the titer of phage recovered after elution measured. Graphs of phage output versus phage input (not shown) were linear with *x* intercepts indicating the fraction of the phage stock that could be selected by panning. Allowing phage

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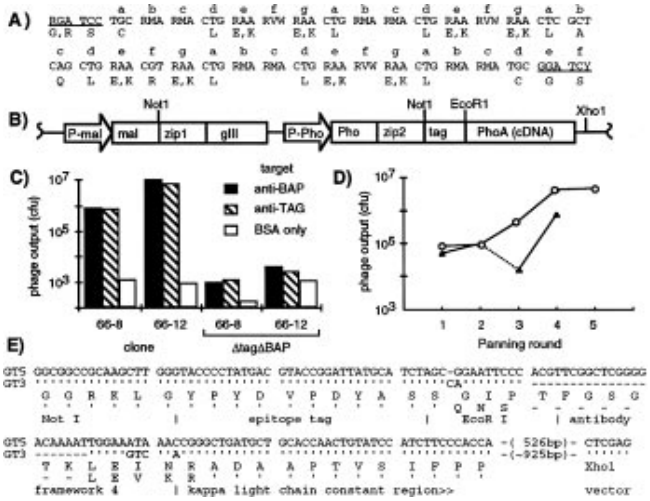

Figure 1. (**A**) Partially degenerate oligonucleotide sequence used to construct zipper libraries. R = A or G; M = A or C; V = A, C or G; W = A or T; RMA codes for A, E, K or T; RVW codes for A, D, E, G, K, R, S or T. (**B**) Schematic of zipper display insert in phagemid pBluescript SK(–). P is promoter; mal, malE (codon –26 to +4) export signal; gIII, c-terminal gIII (248–406) fragment; Pho, Pho A (–21 to +4) export signal; tag, epitope (YPYDVPDYA); PhoA, gene coding for BAP (1–449). (**C**) Assay for functional zippers by test panning of individual phage clones and derivatives with the epitope and BAP deleted against antibodies specific for BAP, the epitope tag, or no antibody. (**D**) Total number of phage output with each panning round for zipper selection (triangle) or display of mouse Fab from cDNA library (circle). The dotted line indicates addition of DTT to washing steps in the zipper selection. (**E**) Sequences of cDNA inserts in clones GT5 (DDBJ/EMBL/GenBank U70311) and GT3. Apostrophes indicate sequence homolgy and dashes represent deletions.

to bind for 16 h at 4°C before washing let 1/70 of the initially applied phage be recovered, ~40-fold better than only binding for 2 h at 37°C, and 10^3–10^4 better than control phage. Comparing p.f.u. to c.f.u. indicated that >90% of the hybrid phage contain phagemid over helper phage genomes. Replacement of BAP sequence in the vector 66-12 and vectors with +1 and +2 frameshifts with A20 (14) cDNA gave a library of phage that display proteins from translated B cell cDNA.

Phage displaying expressed B cell proteins were panned against antibodies specific for mouse Fab. During five rounds; the number of phage retained in each step increased with each round as is expected as the library becomes enriched for binding sequences (Fig. 1D, circle). Furthermore, restriction digests of library plasmid at each step showed that two inserts predominated in the library after the third panning round. These two clones GT3, a 950 bp insert, and GT5 (DDBJ/EMBL/GenBank U70311), a 550 bp insert, were sequenced and found to be 98% homologous to kappa light chain constant region (15; DDBJ/EMBL/GenBank X56394) fused in frame to the epitope tag, but the clones differ in reading frame, 5′ ends, and length of 3′ untranslated sequence (Fig. 1E).

While the results are very exciting and demonstrate the potential of the phage display expression cloning technique, one can anticipate some limitations on the types of proteins that could be recovered using this method. Successful display requires that the protein be expressed in *Escherichia coli*, translocate to the periplasm, fold correctly, and be incorporated into the phage particles. Sequences that interfere with any of these steps could reduce or eliminate panning efficiency. Selecting for binding affinities that require post-translational modification of the displayed protein, such as phosphorylation, would require *in vitro* modification of the displayed protein to be successful. In spite of these limitations, a large variety of proteins have been displayed on phage (1–2), including antibodies (4), enzymes (10), hormones, lymphokines and DNA binding proteins (16).

We have demonstrated the use of phage display to select a zipper pair from a partially randomized library, and used this zipper to display proteins translated from a cDNA library on phage, which were then selected for display of Fab by panning. From this phage display expression cloning, kappa light chain fragments were recovered and identified by DNA sequence. Speed, sensitivity and efficiency promises to make phage display a valuable technique for cloning cDNA.

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