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Affinity Selection of DNA-Binding Proteins Displayed on Bacteriophage λ

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Two transcription factors, human ATF1, its DNA-binding domain (ATF1BD), and the DNA-binding domain (GAL4BD) of the yeast GAL4 protein, were displayed on the surface of bacteriophage λ vectors and efficiently selected by DNA fragments immobilized in microtiter wells. The DNA-binding proteins are fused to the carboxy terminus of the tail protein gpV and head protein gpD of the vectors, λfoo and λfooDc, respectively. After a single round of affinity selection, the fusion phages were successfully enriched 60- to 4,000-fold over the vector phages. Further, the GAL4BD fusion phages were enriched 5- and 15-fold by affinity selection using specific DNA as probes over nonspecific DNA when expressed on λfooDc and λfoo, respectively. The ATF1BD fusion phages were also sequence-specifically enriched greater than 4-fold when displayed on λfoo. These results suggest that the λfoo display system is useful for *in vitro* studying of protein–DNA interactions and may be applied to screening of DNA-binding protein from complex cDNA libraries through DNA-binding affinity.

Key words: bacteriophage lambda, DNA-binding protein, phage display, protein–DNA interaction, sequence-specific selection.

Protein–DNA interaction plays central roles in many biological phenomena such as mRNA transcription and signal transduction. Therefore, the screening and determination of proteins and functional domains having DNA-binding activity is of great interest. However, existing approaches, including analysis of the yeast one-hybrid system or Southwestern blotting, are not powerful enough to systematically acquire information on functional DNA-binding proteins or domains. Phage display is a useful means of identifying and selecting molecules of interest that bind to specific targets. Intensive analyses of phage-displayed peptides or proteins have been carried out with the filamentous phage M13 (*1–3*). Random peptide libraries of M13 phage display have been used for selecting zinc fingers using the new DNA-binding specificities of the Cys_2-His_2 class peptide, Zif268 (*4–6*). Apart from the Cys_2-His_2 zinc finger phage, there have been few reports describing the applications of phage-displayed proteins to DNA affinity selection. To our knowledge, only one approach using DNA-based selection from peptide libraries displayed on the surface of M13 has been reported for identifying DNA-binding motifs capable of discriminating single-stranded from double-stranded oligos irrespective of sequence (*7*). This approach failed to identify the phage by using sequence-specific binding affinity. Otherwise, selection methodologies have relied almost exclusively on highly specific affinity selection such as immunological recognition of proteins. In spite of the wide use of filamentous phage display systems, the problem remains that the protein to be displayed must translocate across the bacterial cytoplasmic membrane. As previously reported, the secretion of cytoplasmic protein is often difficult, even if it is fused to a bacterial signal peptide or a naturally secreted protein (*8–10*). Also, water-soluble peptides and proteins fused to the phage coat protein may interfere with the passage of the fusion product from the cytoplasm to the periplasm (*11–13*). The incompetence of the proteins for secretion decreases the possibility for a variety of proteins to be displayed.

Bacteriophage λ vectors have recently been introduced as alternative display vehicles (*14–18*). One great advantage of the λ system compared to M13 is that, since the phage assembles in the bacterial cytoplasm before its release from the cell, there is no requirement for the displayed peptides or proteins to be secreted. In the λ phage vector, λfoo, foreign proteins are fused to the C-terminus of the tail protein, gpV, through a suppressible amber codon in the linker arm that allows the conditional fusion of cloned sequences. Thus, the number of fusion proteins per phage particle is controllable by the suppressor activities of *Escherichia coli* hosts. This also overcomes the toxic effects of the displayed protein on viability of the phage and *E. coli* host. Further, the ribosome-binding site in the linker region between the phage coat and foreign proteins allows initiation of unfused proteins as separate products and leads to assembly of a homomultimeric protein. Using this vector, multimeric *E. coli* β-galactosidase (β-gal) and the plant lectin *Bauhinia purpurea* agglutinin (BPA) were successfully displayed and purified by affinity selection (*14*). In another λ vector, λfooDc, fusions are made to the C-terminus of the capsid protein, gpD. Some peptides or proteins, including IgG-binding domains of *Staphylococcus aureus* protein A, and

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β-lactamase and β-gal expressed on the λ fooDc phage were shown to be functionally active (*15*, *17*). The fusion phages were successfully enriched through antibody–antigen or lectin–sugar interaction, one of the strongest and most specific biomolecular interactions.

To examine the feasibility of using λ phage vectors for displaying DNA-binding proteins and to select fusion phages by target DNA sequences, we chose two eukaryotic transcription factors, yeast GAL4 and human ATF1, as model DNA-binding proteins to assess the potential of lambda vectors for genome-wide screening. GAL4 and ATF1 are well-studied major classes of transcription regulators and have different DNA-binding motifs, the C_6 zinc cluster (GAL4) and the leucine zipper (ATF1) (*19*, *20*). To the best of our knowledge, neither C_6 zinc cluster- nor leucine zipper-containing DNA-binding proteins have been displayed *via* any phage vectors used for displaying peptides or proteins. The DNA-binding domain (1–147 aa) of GAL4 contains a $Zn(II)_2Cys_6$ motif in a binuclear complex with two Zn(II) or Cd(II) ions (*21*, *22*). The protein binds as a dimer to a 17-bp palindromic DNA sequence, 5′-CGG-N_6(T/A)N_6CCG, which is observed in the galactose-inducible upstream activating sequence (UAS_G) (*19*, *23*, *24*). The ATF family is involved in cAMP- and calcium-induced regulation during transcription (*20*, *25*, *26*). ATF1 binds to DNA as a dimer, and the consensus binding site 5′-TGACGTCA is present in many viral and cellular promoters (*27*). The DNA-binding and dimerization domain of ATF1 is comprised of a carboxy-terminal basic leucine zipper (bZIP) motif. All ATF family members are significantly similar to one another within this region (*28*, *29*).

In this work, we have demonstrated the effectiveness of two λ vectors to display and select functionally active DNA-binding proteins, GAL4 and ATF1. The success of this approach may allow us to isolate phage clones expressing DNA-binding proteins from diverse cDNA or genomic DNA libraries, and to extend the study of protein–DNA interactions.

MATERIALS AND METHODS

Cloning of Genes into λ Vectors—The gene encoding GAL4BD (DNA-binding domain of GAL4) was obtained from plasmid pCL1 (*30*) by PCR amplification, using primers 5′-ACGTCAAAGCTTGAAGCTACTGTCTTCTATC and 5′-CCCCGGGAATTCTTACTCCATGGCCAT. These primers contain a restriction site, *Eco*RI or *Hin*dIII (underlined), and a stop codon (doubly underlined). cDNAs encoding ATF1 and ATF1BD (the DNA-binding domain of ATF1) were isolated from Superscript™ human brain cDNA library (Gibco BRL, Rockville, MD) by PCR amplification, using primers 5′-ACAGTTAAGCTTGGAAGATTCCCACAAGAG, 5′-AGTACAAGCTTGGGTACAACTATTCTTCAGTAT and 5′-CTTTCTGAATTCTCAAACACTTTTATTGGA, respectively. The amplified DNAs were digested with *Hin*dIII and *Eco*RI restriction enzymes and cloned into the plasmid pBluescript II KS+ (pBS) (Stratagene, La Jolla, CA). DNA sequencing was carried out to confirm that all the PCR products had the correct sequences.

Bacteriophage λ vectors λfoo and λfooDc were purified according to standard procedures, and digested with *Hin*dIII and *Eco*RI. DNA inserts encoding GAL4BD, ATF1, and ATF1BD were excised from the pBS constructs with *Hin*dIII and *Eco*RI and ligated to either λfoo (yielding λV-GAL4BD, λV-ATF1, and λV-ATF1BD, respectively) or λfooDc (yielding λD-GAL4BD, λD-ATF1, and λD-ATF1BD, respectively) arms. The ligated DNA was packaged using Gigapack III Gold Packaging Extract (Stratagene), and plated after infecting *E. coli* JM105 strain (*sup*^{0}) as described by Maruyama *et al.* (*14*). The recombinant phages were isolated, confirmed by plaque PCR, then amplified by infecting *E. coli* Q447 (*sup*^{0}). Protein display on the phage surface was achieved by infecting *E. coli* strains, Q358 (*supE*) and XL1-Blue (*supE*).

Western Blotting Analysis and Quantitation of Fusion Proteins—General manipulations of phage to express fusion proteins on the phage surface were carried out as described by Maruyama *et al.* (*14*) with some modifications. Fusion phages grown in 5 ml of *E. coli* host cell culture were precipitated by adding polyethylene glycol (PEG) 8000 (Sigma, St. Louis, MI) to 6% (w/v) and NaCl to 0.6 M at final concentrations. Pellets were resuspended in 200 μl of λ dilution buffer and centrifuged once to remove the undissolved pellet. An aliquot of approximately 1×10^{10} pfu (plaque formation units) of phages was denatured in sample buffer [12 mM Tris-HCl (pH 6.8), 0.4% SDS, 2.88 mM β-mercaptoethanol, 5% glycerol, and 0.02% bromophenol blue] for 5 min at 100°C, subjected to SDS/12.5% polyacrylamide gel electrophoresis, and then either stained with Coomassie Blue or blotted onto a PVDF membrane (BioRad, Hercules, CA). After blocking with blocking buffer [10 mM Tris pH 7.5, 150 mM NaCl, 1% BSA (Sigma), 1% skim milk] overnight at 4°C, the blot was incubated for 1 h with primary antibody, either mouse anti-GAL4BD monoclonal antibody (12.5 μg/ml, Santa Cruz Biotechnology, South Cruz, CA) or mouse anti-ATF1 monoclonal antibody (12.5 μg/ml, Santa Cruz), then for 2 h with secondary antibody, horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG (Santa Cruz). Proteins were detected by means of ECL detection reagent (Amersham, Bucks, England) for chemiluminescent reaction followed by exposure to X-ray film (Kodak, Rochester, NY). The amounts of GAL4BD and ATF1 expressed on the phage surface were quantitated using the PhosphorImaging System with Molecular Analyst 2.1 software (BioRad). As a standard protein, polyhistidine-tagged ATF1 protein (Santa Cruz) ($r^2 = 0.997$) or GST-fused GAL4BD protein (Santa Cruz) ($r^2 = 0.996$) was used.

Preparation of DNA Fragments for Affinity Selection—To construct GAL4BD-specific target DNA, an oligonucleotide (5′-GATCCGACGGAAGACTCTCCTCCGTGCGTCCTAG) containing the GAL4-binding site (underlined) was annealed to its complementary strand and concatenated at the *Bam*HI site (dotted underline) by T4 DNA ligase. After size-fractionation by polyacrylamide gel electrophoresis, the concatenated mixture was cloned into the *Bam*HI site of pBS. The plasmid, containing 8 repeats of the GAL4-binding site, was screened and used as a template for PCR with biotinylated and unbiotinylated primers to amplify the 550-bp fragment as the GAL4 target sequence. An approximately 650-bp DNA fragment free of the GAL4-binding site was used as nonspecific DNA for GAL4BD. In addition, a 36-mer fragment (5′-CCGATCCGACGGAAGACTCTCCTCCGTGCGTCCTCG) and 60-mer fragment (5′-GCGATCCGACGGAAGACTCTCCTCCGTGCGTCCATCCGACGGAAGACTCTCCTCCGTGCG) containing a single and two re-

peats of the GAL4-binding site, respectively, were also constructed.

A 60-mer oligonucleotide [5′-GGGCT(CTGACGTCAGCC-AT)$_4$CCAT] containing four ATF1-binding sites (underlined) derived from rat somatostatin CRE was biotinylated at the 5′ end and annealed to its unlabeled complementary strand, yielding AS60. As nonspecific DNA (AN60), the four ATF1-binding sites were replaced with GGACTTAC, CGACGACA, AGACGTCA, and TGTCGTCA. A 36-mer oligonucleotide containing the ATF-binding site (5′-GATCCGGCGACTCCTTGGATGACGTCATACAGATAG) and biotinylated 380-bp ATF1-binding fragment containing 4 repeats of the ATF1-binding site (AS380) were also obtained as described above in the preparation of the GAL4-binding target. As nonspecific DNA for ATF1, a fragment with the same length, but free of ATF1-binding sites (AN380) was constructed by replacing the ATF1-binding site (TGACGTCA) with AGACTTAC.

Affinity Selection of Fusion Phage—Fusion phages amplified by infection of *E. coli* Q447 were transferred to 20 ml of CY medium supplemented with 50 μM $ZnCl_2$ for the GAL4BD fusion phage and infected into XL1-Blue to produce the fusion protein. After amplification, phage particles were purified by PEG precipitation and resuspended in 200 μl of phage binding buffer [buffer for the GAL4BD fusion phage contained 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 μM $ZnCl_2$, 100 mM NaCl, 5% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 2 μg/μl Ac-BSA (Sigma), and 0.05 μg/μl poly dI-dC (Promega, Madison, WI); buffer for the ATF1 fusion phage contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, 2 μg/μl Ac-BSA, and 0.05 μg/μl poly dI-dC]. λfoo or λfooDc vector phage was also amplified at the same time for use as a negative control for panning.

The biotinylated DNA constructed above as an affinity probe was dissolved in DNA-binding buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% BSA] and the solution (100 μl each) was added to each well of a streptavidin-coated polystyrene plate (Pierce, Rockford, IL) at a final concentration of 1.25 μM. Binding was carried out for 2 h at 37°C with gentle shaking. Unbound DNA fragments were removed by washing three times with 300 μl of DNA-binding buffer containing 0.05% Tween-20.

To select the GAL4BD, ATF1, or ATF1BD fusion phage, the microtiter wells coated with DNA fragments were washed with phage binding buffer, loaded with a mixture (100 μl) of the fusion and corresponding vector phages, and incubated for 1 h at 25°C. Unbound phages were removed by washing 10 times with washing buffer (phage binding buffer supplemented with 0.05% Tween-20). Bound phages were eluted from the wells with 100 μl of λ dilution buffer containing 2 M NaCl for 30 min at 25°C. The eluted phages were immediately plated with JM105 onto a λ plate using λ top agar containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Titers of fusion and vector phages were scored from the number of white and blue plaques, respectively, to calculate the enrichment factors for DNA affinity selection.

RESULTS

Display of DNA-Binding Proteins on λ Phage—A DNA fragment encoding *S. cerevisiae* GAL4 DNA-binding and dimerization domains (GAL4BD; 1–147 aa), full-length human ATF1 (1–271 aa), or ATF1 DNA-binding domain containing the basic region and the leucine zipper motifs (ATF1BD; 146–271 aa) (*25*) was PCR-amplified and cloned into *Eco*RI and *Hin*dIII sites on λfoo or λfooDc as described in "MATERIALS AND METHODS." To confirm the expression of DNA-binding proteins on the phage surface, the phages were cultivated with suppressor-positive *E. coli* such as XL1-Blue or Q358, and phage proteins were analyzed by Western blotting with specific antibodies after PEG precipitation of phages from their liquid cultures. The gpD-GAL4BD fusion protein and unfused free GAL4BD protein migrated as bands with molecular masses of 35 and 19 kDa, respectively, which are in good agreement with the calculated molecular masses (Fig. 1A, lanes 1–4). However, the molecular masses of the gpV-GAL4BD fusion protein and unfused free GAL4BD protein were 54 and 25 kDa, approximately 12 and 6 kDa larger than those calculated, respectively (Fig. 1A, lanes 5–8). The larger molecular weight of the fused gpV-GAL4BD is probably due to the unusual mobility of gpV as reported by Katsura (*31*) and Dunn (*16*). However, it is not clear why the mobility of unfused GAL4BD expressed by λV-GAL4BD was decreased. No mutations were detected by the DNA sequencing of the

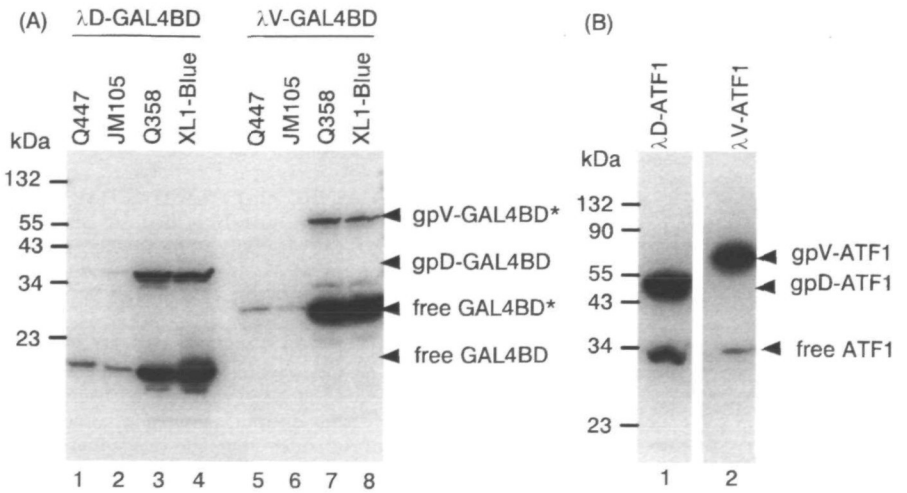

Fig. 1. **Western blotting of GAL4BD and ATF1 proteins incorporated into phage particles.** The GAL4BD fusion phages grown with the indicated *E. coli* strains and the ATF1 fusion phages grown with XL1-Blue were partially purified by PEG precipitation. Approximately 1×10^{10} pfu of the GAL4BD fusion phages (A) or 1×10^{8} pfu of the ATF1 fusion phages (B) were loaded per lane on 12.5 or 10% gel, respectively. After gel electrophoresis, phage proteins were blotted and stained with anti-GAL4BD or anti-ATF1 antibodies as described in "MATERIALS AND METHODS." The arrowheads indicate the positions of the fusion and free proteins. Asterisks indicate that proteins migrated at unexpectedly slower rates (refer to text).

λV-GAL4BD in the region encompassing the gpV gene to the end of GAL4BD. A possible explanation is that the free GAL4BD translation may initiate at somepoint upstream of the translation initiation site in the linker region. There is a possible translation initiation site having a potential Shine-Dalgarno consensus within the gene *V*. The translation initiation efficiency depends not only on nucleotide sequence but also on mRNA conformation (*32*). The insertion of GAL4BD DNA might cause a conformational change of λ mRNA and activate this type of inactive translation initiation site, yielding a GAL4BD product 6 kDa larger than expected. When cultivating the λ constructs with *sup*^{0} strains Q447 and JM105, the fusion product gpV-GAL4BD was not detected (Fig. 1A, lanes 1, 2, 5, and 6). The ratio of the fused and free GAL4BDs should be 1:1 based on the dimeric structure of the protein. Western blotting analysis indicated that free GAL4BD had been supplied in sufficient quantity for the fusion phages to bind DNA (Fig. 1A, lanes 1–8). The ATF1 fusion proteins with molecular masses of 50 and 68 kDa were detected in λD-ATF1 and λV-ATF1 preparations, respectively, when cultivated with XL1-Blue (Fig. 1B, lanes 1 and 2). Free ATF1 proteins from both gpD and gpV fusion phages were detected as bands with the molecular masses of 32 kDa, which was in good agreement with the calculated molecular mass. Because antibody against the DNA-binding domain of ATF1 is not available, ATF1BD expression could not be detected by Western blotting.

To examine the display efficiency of fusion proteins, the fusion products were quantitated from the band intensity on the Western blot by comparing with those of the standard proteins (Table I). The result indicated that GAL4BD was incorporated into approximately 16% of total gpV and 50% of total gpD of λ phage particles when grown on Q358 with a strong suppressor activity. In the case of ATF1, 33% of total gpV and 68% of total gpD were fused for λV-ATF1 and on λD-ATF1, respectively, using the same host.

Affinity Selection of DNA-Binding Fusion Phages—Microtiter plates coated with various biotinylated DNA fragments were used for the enrichment of fusion phages. Fragments of 550 bp (GS550), 60 bp (GS60), and 36 bp (GS36) containing 8, 2, and 1 repeat of the GAL4-binding site, respectively, were used as sequence-specific DNAs. λV-GAL4BD and λD-GAL4BD grown with XL1-Blue were mixed with the vector phage λfoo and λfooDc, respectively, and then subjected to a single round of affinity selection. As shown in Fig. 2, the GAL4BD fusion phages were recovered more efficiently than the vector phages by using DNA fragments of any length. The most efficient enrichment was achieved with the GS550 DNA for both of the fusion phages, although shorter DNA fragments could also be used for successful affinity selection. We therefore used the GS550 DNA as a specific probe in panning experiments for the GAL4BD fusion phages.

To examine how efficiently λV-GAL4BD and λD-GAL4BD fusion phages can be enriched over the vector phages by sequence-specific DNA-affinity selection, mixtures of the fusion and vector phages at various input ratios were subjected to a single round of affinity selection. As shown in Table II, the enrichment factors of λV-GAL4BD and λD-GAL4BD were enriched 4.4×10^3- and 3.0×10^3-fold, respectively, by using the DNA fragment containing the GAL4-consensus. The values were approximately 3 to 5 times higher than those for the DNA fragment lacking the consensus. These results indicate that GAL4BD-displaying phages were sequence specifically enriched with GAL4-binding sequence. The increased recovery of the λfooDc-based fusion phage is possibly due to the high expression level of GAL4BD (Talbe I). However, the increased non-specific binding of the vector λfooDc decreased the efficiency of λD-GAL4BD enrichment. As a result, the best enrichment was achieved by using λfoo in spite of the less efficient display of the fused proteins in comparison with λfooDc. The GAL4BD fusion phages were also enriched to some extent in the absence of coated DNA (Table II). Peptide chains folded inside the native protein molecule may be exposed on the surface of the displayed protein whose polypeptide was truncated for the fusion construction, resulting in the increase of non-specific interaction with avidin and/or plastic surfaces.

For affinity selection of the ATF1 fusion phage, a 60-bp DNA fragment containing an ATF1-binding site, TGACGTCA, (AS60) and 60-bp DNA fragment free of ATF1-binding consensus (AN60) were prepared as described in "MATERIALS AND METHODS." As in the GAL4BD experiments, mixtures at various ratios of the fusion and vector phages grown with XL1-Blue were subjected to a single round of affinity selection. Although the ATF1-displaying phages

TABLE I. **Amount of GAL4BD and ATF1 displayed on the surface of λfoo and λfooDc.** Fusion phages grown with Q358 were purified by PEG precipitation and then subjected to Western blotting. The numbers of GAL4BD and ATF1 molecules displayed were quantitated from the data shown in Fig. 1 as described in "MATERIALS AND METHODS."

Vector	Fused protein	Copies/ virion[a]	Displayed molecules/virion		
			GAL4BD	ATF1	β-Galactosidase
λfoo	gpV	192	31.3	63.1	0.5[b]
λfooDc	gpD	420	220.3	287.3	34.3[c]

[a,b,c]Data from Ref. *35*, *14*, and *17*, respectively.

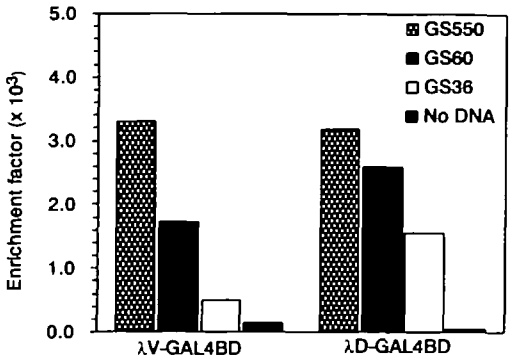

Fig. 2. **Enrichment of λV-GAL4BD and λD-GAL4BD by DNAs containing various repeats of the binding site.** 10^7 pfu of λV-GAL4BD and λD-GAL4BD grown with XL1-Blue were mixed with 10^{10} pfu of vector phages, λfoo and λfooDc, respectively, and selected through a single round of panning with immobilized DNA fragments, GS550 (550 bp), GS60 (60 bp), and GS36 (36 bp) containing 8, 2, and 1 repeat of the GAL4-binding site, respectively, as described in "MATERIALS AND METHODS." Eluted phages were plated after infecting JM105 in the presence of X-gal and titered by counting blue and white plaques for vector and fusion phages, respectively. Enrichment factors were calculated based on the ratio of output ratio (λV-GAL4BD/λfoo or λD-GAL4BD/λfooDc) to input ratio (λV-GAL4BD/λfoo or λD-GAL4BD/λfooDc).

TABLE II. **DNA-affinity enrichment of fusion phages.** The fusion phages were mixed with corresponding vector phages and affinity-selected with either specific DNA (GS550 for GAL4BD, AS60 for ATF1, and AS380 for ATF1BD) or non-specific DNA (GN650 for GAL4BD, AN60 for ATF1, and AN380 for ATF1BD). The enrichment factor was calculated based on the ratio of output ratio to input ratio as described in Fig. 2, and represents the average ± SD of three independent experiments.

Phage	Input phage (pfu) (Fusion/vector)	Enrichment factor ($\times 10^2$)*		
		Specific DNA	Non-specific DNA	No DNA
λV-GAL4BD	$2.6 \times 10^7/1.1 \times 10^{10}$	44 ± 11 (1.5/0.00034)	3.0 ± 1.0 (0.16/0.00054)	1.2 ± 0.6 (0.071/0.00058)
λD-GAL4BD	$4.6 \times 10^7/1.4 \times 10^{10}$	30 ± 9.4 (7.6/0.0025)	8.0 ± 0.3 (2.2/0.0027)	0.9 ± 0.2 (0.27/0.003)
λV-ATF1	$4.9 \times 10^9/3.2 \times 10^{11}$	2.4 ± 1.3 (0.015/0.000062)	1.8 ± 0.78 (0.0066/0.000036)	1.5 ± 0.89 (0.0067/0.000043)
λD-ATF1	$2.3 \times 10^9/3.0 \times 10^{10}$	0.99 ± 0.29 (0.027/0.00027)	1.2 ± 0.41 (0.038/0.00034)	0.74 ± 0.40 (0.021/0.00028)
λV-ATF1BD	$1.4 \times 10^7/1.6 \times 10^9$	0.59 ± 0.29 (0.23/0.0038)	0.13 ± 0.05 (0 12/0.0082)	0.06 ± 0.01 (0.04/0.0066)

*The average phage recoveries (%) in the three independent experiments are shown in parenthesis (fusion phage recovery/vector phage recovery) under the corresponding enrichment factor.

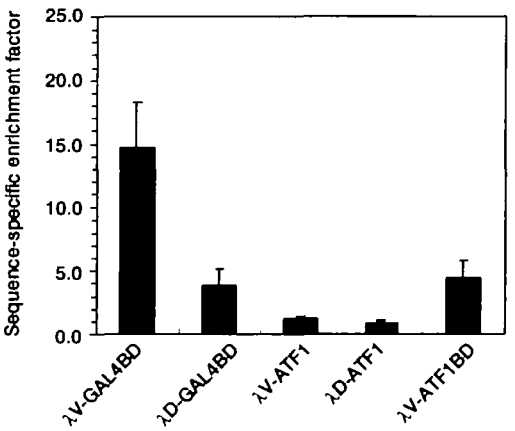

Fig. 3. **Sequence-specific enrichment analysis of various fusion phages.** Sequence-specific enrichment factors were calculated based on the ratio of the enrichment factor with the specific DNA *versus* that with non-specific DNA as shown in Table II. Input phages were the same as indicated in Table II. The data represent the average ± SD of three independent experiments.

appeared to be enriched by DNA affinity, the enrichment was much less effective than the GAL4-displaying phages and no sequence-specificity was observed when ATF1 was fused to gpD. On the other hand, λV-ATF1BD was enriched approximately 10 and 5 times by the specific DNA as compared to the absence of DNA and non-specific DNA, respectively. The AS380 DNA containing 4 repeats of the ATF1-binding sequence was also used for λV-ATF1BD enrichment, but no significant differences between the recoveries after selection with the two DNA fragments (AS60 and AS380) were observed (data not shown). The recovery of both fusion and vector phages appeared to depend on the number of phages loaded in the well, as revealed by λV-ATF1 and λV-ATF1BD experiments. This indicates that there is a limit to the binding capacity for the DNA-independent binding of vector phages, as there is for the DNA-dependent binding of fusion phages. The phage numbers within the range used in this work did not significantly affect the enrichment factors (data not shown). In order to examine whether binding of the fusion phage to DNA is DNA-sequence specific, we calculated the sequence-specific enrichment factors. As shown in Fig. 3, the highest sequence-specific enrichment (15-fold) was observed when GAL4BD was displayed on λfoo through gpV fusion. The sequence-specific enrichment of GAL4BD, however, was significantly reduced (3.8-fold) when displayed on λfooDc through gpD fusion. The sequence-specific enrichment factors of phages displaying ATF1BD through gpV were 4.5.

DISCUSSION

We have succeeded in displaying active DNA-binding domains of GAL4 and ATF1 on the λ phage surface and have demonstrated sequence-specific enrichment of fusion phages. This is the first successful demonstration of enrichment of the functional domain of multi-complex DNA-binding proteins by DNA-affinity selection. Phage displaying GAL4BD was approximately 4,000-fold enriched over the vector phage after a single round of DNA-affinity selection. Originally, we planned to display full-length (intact) DNA-binding proteins using the λ phage display system. The full-length GAL4 fused to either gpV or gpD has been successfully constructed, and was confirmed by plaque PCR (data not shown). Nevertheless, we were unable to amplify the GAL4 fusion phage due to its extremely tiny plaques, irrespective of the suppressor activity of the host cells. Since GAL4 is a yeast protein expressed at only approximately 0.1 copy per cell (*33*), the overexpressed GAL4 might be toxic to *E. coli* by interfering with phage production or cell metabolism (*15*). Alternatively, the introduction of the *GAL4* gene into the phage genome may affect the phage mRNA conformation, interfering with the phage growth.

GAL4BD assembles as dimers to bind its target DNA with higher affinity (*21*). The conditional fusion provided by the λ vectors allows the formation of such dimers, since both fused and unfused subunits are synthesized. Western blotting analysis clearly showed that both fused and unfused free GAL4BD were expressed. The gpD fusion scheme offered more efficient display of the fusion protein than that of the gpV fusion, and better recovery of the fusion phage over the gpV fusion was observed. This would be expected because the copy number per virion of gpD is higher than that of gpV (*34*). On the other hand, more efficient sequence-specific enrichment for the gpV fusion on

λfoo was observed than in the gpD fusion on λfooDc. This is due to the tendency of the λfooDc-based phages to bind DNA regardless of its sequence, as compared to the λfoo-based phages. The artificial modification of gpD and/or higher expression of foreign proteins on λfooDc than on λfoo may enhance the nonspecific binding of the λfooDc-based phages to DNA.

The K_d value for the complex of GAL4BD with a 17-bp recognition site is 20 pM (*20*). K_d for ATF1 has not been measured, but K_d values of many other bZIP proteins are on the nanomolar scale (*25*), which is much higher than those of C_6 zinc cluster proteins. The low recovery of ATF1-displaying phage as compared to GAL4BD-displaying phage may be due to the higher K_d value for DNA of ATF1 than that of GAL4BD. As was observed in the Western blotting experiments, the insufficient amount of free ATF1 protein may be due to insufficient dimerization of ATF1 on the phage surface. This may result in inefficient enrichment of the fusion phage. Enrichment factors were improved when ATF1BD was displayed instead of the full-length ATF1. This implies that ATF1BD is more suitable for expression on the phage than the full-length ATF1. Although the amount of expressed ATF1BD could not be determined, more free ATF1BD molecules than the full-length ATF1 may be supplied for dimerization. Alternatively, the assembly and/or folding of the smaller protein might be more efficient than at for the full-length version.

Our study demonstrates the potential of the λ display system for selecting DNA-binding proteins by affinity interaction. Development of a genome-based field of research requires new technologies for the systematic survey of biomolecular interaction. Protein–DNA interaction is not as strong as protein immunological interaction but is specific enough for systematic screening. This is an important step toward isolation of cDNA encoding DNA-binding proteins or domains from a library. Our results indicate that λ phage display may serve as an efficient means of studying protein–DNA interaction, and of genome-wide screening of DNA-binding transcription factors.

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