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The Use of Polypeptide Probes Selected From Artificial Peptide Libraries for the Recognition and Differentiation of DNA Sequences

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Abstract: In the novel approach suggested here, we examined the use of polypeptide probes selected from artificial peptide libraries for the identification of the differences in dsDNA sequences which are potential targets for the genetic diseases. As a model system two target DNAs which are the polymerase chain reaction (PCR) products of the two phage–display vector multiple cloning sites (MCS) differing at 18 base pair long has been used. In the production of the target sequences biotinylated primers were used in PCR. The polypeptide probes obtained from artificial peptide library by the selection of phage–display techniques. Identified clones bearing the candidate polypeptide probe specific to the target sequences was tested by phage–ELISA. For the comparison and selection assays, magnetic separation technique was used. One clone selected from artificial peptide library specifically recognized the 18 nucleotide difference, which encodes $(His)_6$, in between two model PCR products and showed no cross reaction determined by phage–ELISA. These findings may be interpreted to the usage of the polypeptides for the detection of the anomalies at specific target DNA sequences and be used for the manipulation of the gene expression in different disease models.

Key Words: Phage display, mutation detection, polypeptide probes.

Introduction

The identification and recognition of the differences present in between the DNA sequences under interest is the major task for the investigation at molecular level in different fields of medical sciences. The recognition of DNA sequences has a wide range of applications either for the detection of the target DNA sequences and modulation of the target genes. For example; the presence of various kinds of mutations of β–thalassemia makes the molecular diagnosis of this disorder time–consuming despite the availability of various powerful molecular techniques, complicating the rapid diagnosis (1). Many research and development laboratories show great effort to identify the problem target showing abnormal genetic composition of the genes either cis–and/or trans–acting factors under interest. Most of these techniques use DNA–DNA interaction like polymerase chain reaction (PCR), dot blot hybridization, reverse blotting etc. On the other hand; the modulation of the gene expression becomes important task in tracing the expression of some genes responsible for defined function in case of modelling the protein targets under interest. DNA–DNA interaction based techniques have many technical limiting factors like the T_m of the interacting probe and target DNA sequences. The use of peptide libraries offers a series of powerful approaches for the discovery of new biological and pharmaceutical reagents (2). Peptides offering high affinity for a particular targets can be identified by selecting from the phage display libraries containing numerous number of individual peptide sequences providing a rich source of novel sequences and structures. Combinatorial approaches have recently been extended to the exploration of DNA–binding mofits in proteins permitting new insights into structural requirements for protein–DNA interaction (3). In our approach, that we suggest here, it will promisingly contribute for the establishment of new detection systems for the quick identification of the target DNA sequences and manipulation of the gene expression under interest at DNA level by the use of peptide structures selected from artificial random peptide libraries.

Materials and Methods

Target sequence preparation

The multiple cloning sites of the two phagemid vecors, pCOCK1 and pDUCK1 used in phage–display technology, were modelled as target DNA sequences. These vectors were obtained from Dr. G Himmler (Institute of Applied Microbiology, Vienna Austria). Target dsDNA sequences, differing only 18 bp sequence, were obtained from these two vector DNAs by PCR amplification using specific primers spanning the MCS (*"Multiple Cloning Site"*) of the vector DNAs (Figures 1, 2). PCR was carried out principally according to the method of Saiki et al (4). Each amplification tube contained 50 mM KCl, 10 mM Tris–HCl (pH=8.3), 1.3 mM $MgCl_2$, 2 µM forward primer, 2 µM biotinylated reverse primer, 0.2 mM each dNTP, 0.1 µg of template DNA and 1.0 U of *Taq Polymerase* (Boehringer Mannheim) in a total volume of 50 µl. The PCR cycles were done by Biometra Trio thermocycler. The cycles comprised incubations of 1 min at 95°C, 2 min 55°C and 2 min 72°C. In total, 30 cycles were performed and PCR tubes incubated at 72°C for 10 min. After PCR cycles, the PCR products were checked with 1.2% Agarose Gel and purified with Agarose DNA Purification Kit (Boehringer Mannheim).

Preparation of 16–mer peptide library

The 16–mer artificial peptide library was prepared at Tübitak Marmara Research Center (5). Main sequence was synthesized with Cruachem PS250 DNA Synthesizer and cloned into phagemid vector pCOCK1 as described in figure 3.

Selection of target specific phage–diplayed clones

Phagemid clones displaying specific peptide sequences on their surfaces, were selected by biopanning procedure
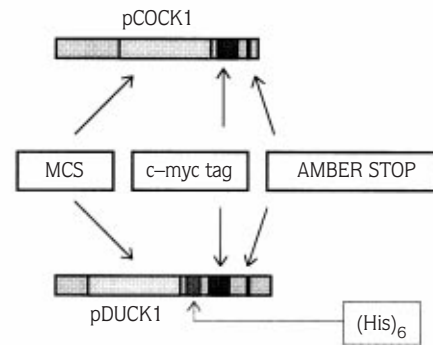

Figure 1. The characteristics of the target DNAs used in the model system (MCS–Multiple cloning site).

(6–9). Since the main aim was to identify the binder peptide sequence specific to the 18 nucleotide present on the pDUCK1 MCS in our model, comparatively selection strategy was followed (Figure 4). Selection was started with the 5'–biotinylated PCR product of pDUCK1. 1 µl of the PCR product was mixed with $1x10^{10}$ tfu phagemid library in a total volume of 100 µl in PBS (phosphate buffered saline), and incubated at 37°C for 30 minutes. After incubation at 37°C, 20 µl streptavidin coated magnetic particles (Dynal) was added into the microcentrifuge tube. Biotinylated PCR product–binded phagemid complex was removed by applying magnetic field and remained unbound phagemids were washed out by PBS. Collected phagemids were eluted by the addition of 100 µl E. Coli (TG1) suspension. Microcentrifuge tube was incubated at 37°C for 1 hour, the elution mix was titrated and amplified to $1x10^{10}$ tfu for the second round of panning. In the second round of panning the collected phagemids were panned against biotinylated PCR product of pCOCK1 to remove phagemids recognizing the regions on the DNA sequence other than 18 bp target sequence encoding for the $(His)_6$. To be able to obtain this result, in

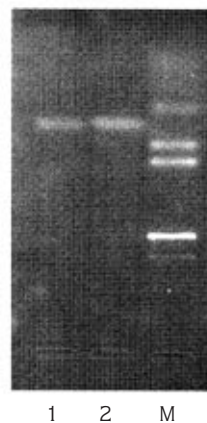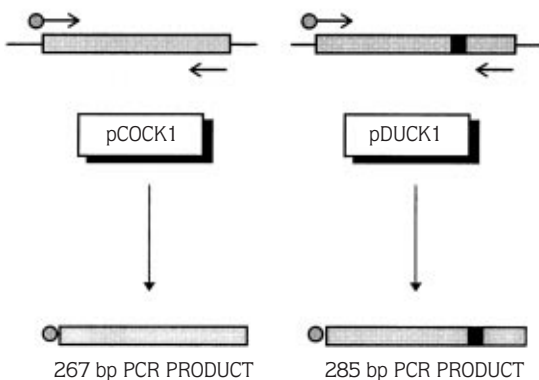267 bp PCR PRODUCT   285 bp PCR PRODUCT

1   2   M

Figure 2. Preparation of target sequences, differing at 18 nucleotides, for phage–displayed artificial library selection. (M: Marker, 1419–517–396–214 bp in size, 1: PCR product of pDUCK1, 2: PCR product of pCOCK1) (●) Biotin (●—➤) Biotinylated 5'–primer (←—) 3'–primer (■) 18 nucleotide sequence encoding $(His)_6$.
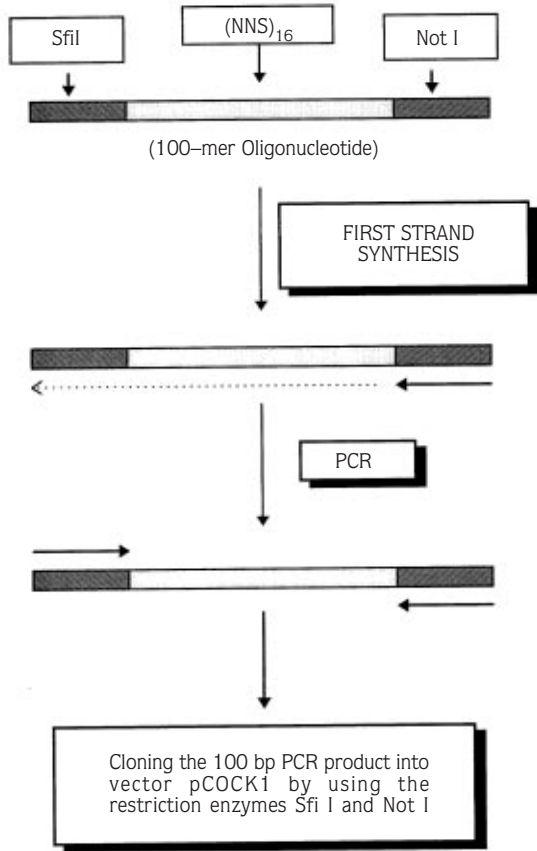
Figure 3.   Preparation of the artificial 16–mer peptide library.
(N → A, C, G, T)
(S → G, C).

the second round of panning unbound phagemids were collected instead of bound phagemids. Unbound phages obtained from the second run were subjected to the third round of panning against biotinylated PCR product of pDUCK1 to enrich the specific phagemids against 18 bp sequence under interest.
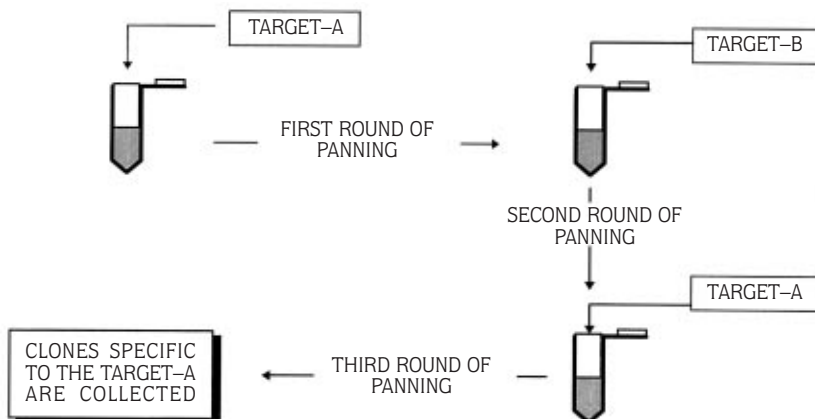
Phage ELISA

The clones obtained after the comparative selection, were subjected to Phage–ELISA according to the modified procedure of Clackson et al (10) as described in figure 5. The positive clones selected by phage–ELISA were accepted as binder phagemids displaying specific peptide sequences on their surface fused to protein 3. Single colonies were inoculated into 200 µl of 2xTY–Tet[6] and grown with shaking for 20–24 hours at 37°C in 96–well PVC ELISA plate. Following centrifugation 25 µl of each supernatant was transferred to the 500 µl microcentrifuge tubes. 2 µl of target sequences which are PCR products carrying biotin added to the supernatants under interest and the binder phagemid–target complexes were collected with streptavidin–coated magnetic particles (Dynal) and detected by anti–pCOCK1 antibody AP (alkaline phosphatase) conjugate according to the procedure given by McCafferty et al (14).

Results

Target sequences were prepared by PCR primers corresponding to the multiple cloning sites (MCS) of the vectors pCOCK1 and pDUCK1 vector DNAs differing at 18 nucleotides (Figure 2). To be able to determine the clones recognizing the 18–nucleotide difference, 16–mer artificial peptide library prepared in our group was interacted with the target DNA sequences by the method of bio–panning as described in method section.

After three rounds of comparative selection, the specific DNA binding clones were reselected by phage–ELISA, as described in Materials and Methods (figures 4, 5). Table 1 shows the phage ELISA results belonging 10 clones out 1000 clones selected. In total 3 clones accepted as positive and coded as EPIDUCK03, EPIDUCK05 and EPIDUCK08 which give OD_{405} values of

Figure 4.   Comparative selection strategy of specific binding phagemid clones.
Target–A: 5'–Biotinylated PCR product of pDUCK1
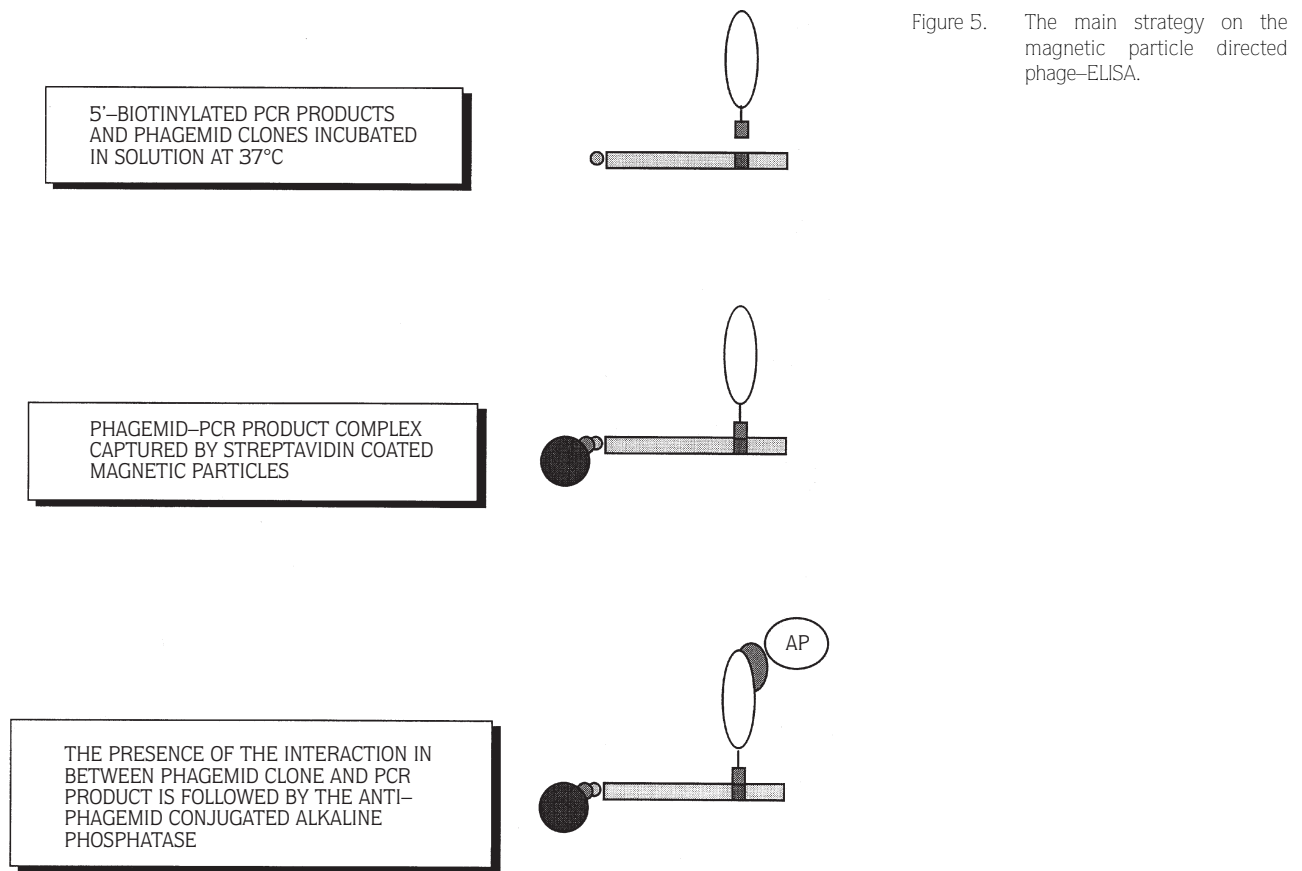Target–B: 5'–Biotinylated PCR product of pCOCK1

Figure 5. The main strategy on the magnetic particle directed phage–ELISA.

1.043, 1.044 and 1.739 respectively. Table 2 shows the phage–ELISA results for the possible cross–reactivity in between two target sequences under interest, and no cross reaction was observed with the clone named EPIDUCK08. The other two clones, EPIDUCK03 and EPIDUCK05 gave high OD_{405} values against the target DNA sequence which do not contain $(His)_6$ coding nucleotides, compared to EPIDUCK08. The cross–reaction experiment was repeated with the clone EPIDUCK08 by phage–ELISA and obtained the same results.

Discussion

EPIDUCK03 and EPIDUCK05 binds to the target–A, pCOCK1–MCS PCR product. But EPIDUCK08 binds to the target–B, pDUCK1–MCS PCR product much more efficiently (table 2). According to these data, EPIDUCK08 shows high OD_{405} values against target–B, pDUCK1–MCS PCR product compared to the target–A, pCOCK1–MCS PCR product and wild type pCOCK1 shows background level compared to the negative control well.

Table 1. ELISA results for the selected clones after three rounds of selection.

CLONES	OD_{405}
EPIDUCK01	0.329
EPIDUCK02	0.740
EPIDUCK03	1.043
EPIDUCK04	0.385
EPIDUCK05	1.044
EPIDUCK06	0.282
EPIDUCK07	0.738
EPIDUCK08	1.739
EPIDUCK09	0.161
EPIDUCK10	0.121
Negative Control[*]	0.077

[*] Negative Control: No target DNA.

Our results confirm the use of peptide libraries offering a series of powerful approaches for the discovery of new biological and pharmaceutical reagents stated by

Table 2.   ELISA results of the positive clones for the cross–reactivity in between two target sequences.

CLONES	TARGET–A	TARGET–B
EPIDUCK03	0.838	1.088
EPIDUCK05	0.920	1.200
EPIDUCK08	0.280	1.388

TARGET–A: pCOCK1–MCS PCR product
TARGET–B: pDUCK1–MCS PCR product
Negative Control: No target DNA (0.075)

Devlin et al (2). Since the recombinant antibody technology with its applications provides a very diverse range of subjects like in health, agricultural and environmental sciences (12), the use of artificial peptide libraries offering high affinity for a particular targets identified by selecting from the phage display libraries containing numerous number of individual peptide sequences providing a rich source of novel sequences and structures can be used in different fields of science while considering the DNA sequences as specific targets under interest. Combinatorial approaches have recently been extended to the exploration of DNA–binding motifs in proteins permitting new insights into structural requirements for protein–DNA interaction (3). In our case; phagemid clone, EPIDUCK08, recognized the target sequence much more efficiently in solution compared to the immobilized state, due to possible conformational changes occurred (data not shown). In our case, the selected phagemid clone can discriminate the difference in 18 nucleotides present in between two, 267 and 285 bp in size, double stranded sequences. On the other hand, the binding experiment was carried under 37°C which means that multiple probing is possible with different peptide probes, in case of the identification of the different problem targets on DNA like different type of deletions, frameshifts and/or mutations responsible for the observed function of thalassemias and abnormal hemoglobins. The novel approach that we suggest here will promisingly contribute the establishment of new detection systems for the quick identification of the differences of DNA sequences under interest and possible manipulation of gene expression at DNA level by the use of peptide structures selected from artificial random peptide libraries.

Acknowledgements

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