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In Vitro Selection of DNA to Chloroaromatics Using Magnetic Microbead-Based Affinity Separation and Fluorescence Detection

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***In vitro* selection (SELEX) of DNA ligands to the chloroaromatics, 4-chloroaniline (4-CA), 2,4,6-trichloroaniline (TCA) and pentachlorophenol (PCP), was performed by a novel method utilizing magnetic beads (MBs) having a linker arm for immobilization. Use of MBs was advantageous in obviating elution and precipitation of DNA as conjugated MBs with surface-captured template DNA could be directly added to PCR mixtures. In addition, a simplified PCR scheme requiring only one type of primer and a rapid fluorescence microscopic method for assessing nucleic acid binding after each round of SELEX were demonstrated.** © 1997 Academic Press

SELEX (Systematic Evolution of Ligands by EXponential enrichment) is a technique developed by Tuerk and Gold (1) to produce specific high affinity oligonucleotide "ligands" to target molecules (Fig. 1). The process involves generation of a highly diverse library of oligonucleotides by randomly mixing equimolar amounts of the four nucleotides at each locus during synthesis. Subsequently, the random library is screened by affinity (column) chromatography and selected (bound) oligonucleotides are eluted by water or free antiligand, concentrated by ethanol precipitation and polymerase chain reaction (PCR) amplified via flanking primer sequences (1-7). The entire process is typically reiterated several times to yield the highest affinity subset of oligonucleotides from the library. SELEX holds several major advantages over antibody technology such as obviating of animal immunization hosts and selection of ligands from far more diverse libraries than is possible with antibody technology (1-3). For example, 10^9 to 10^{11} murine immunoglobulin types are possible (3), while the diversity of an oligonucleotide library is: 4^n or $10^{n\log 4}$, where n is the length of the oligonucleotide (2, 3). Thus, a random 30-mer potentially represents roughly 10^{18} different nucleotide sequences or candidate ligands from which to select a high affinity fraction.

In recent years SELEX has been used to generate specific high affinity oligonucleotides to a variety of clinically important proteins (2) and small molecules (2-7) for diagnostic and potentially therapeutic purposes. Little or no attention has been given to the prospect of using SELEX to generate novel nucleic acid ligands for capture, concentration, detection, or potential remediation of organic pollutants in the environment.

The present report describes application of SELEX technology to the generation of nucleic acid ligands for chloroaromatics; an important class of environmental pollutants. Novel and advantageous aspects of the method chosen include the use of analyte (antiligand) immobilization on MBs for affinity separation, a simpli-

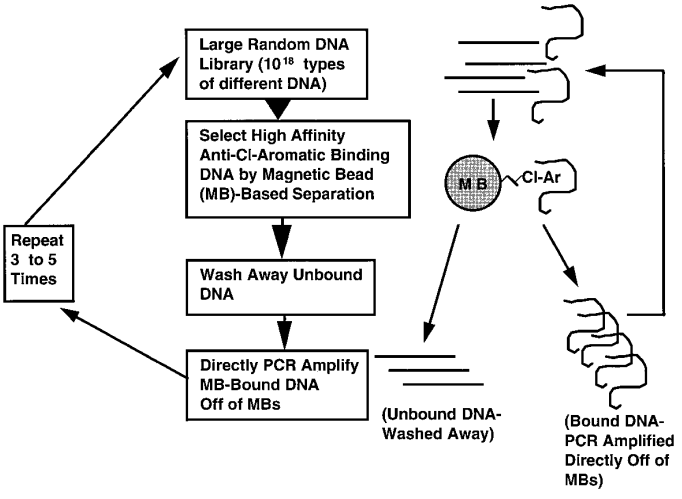

FIG. 1. Modified MB-SELEX method used.

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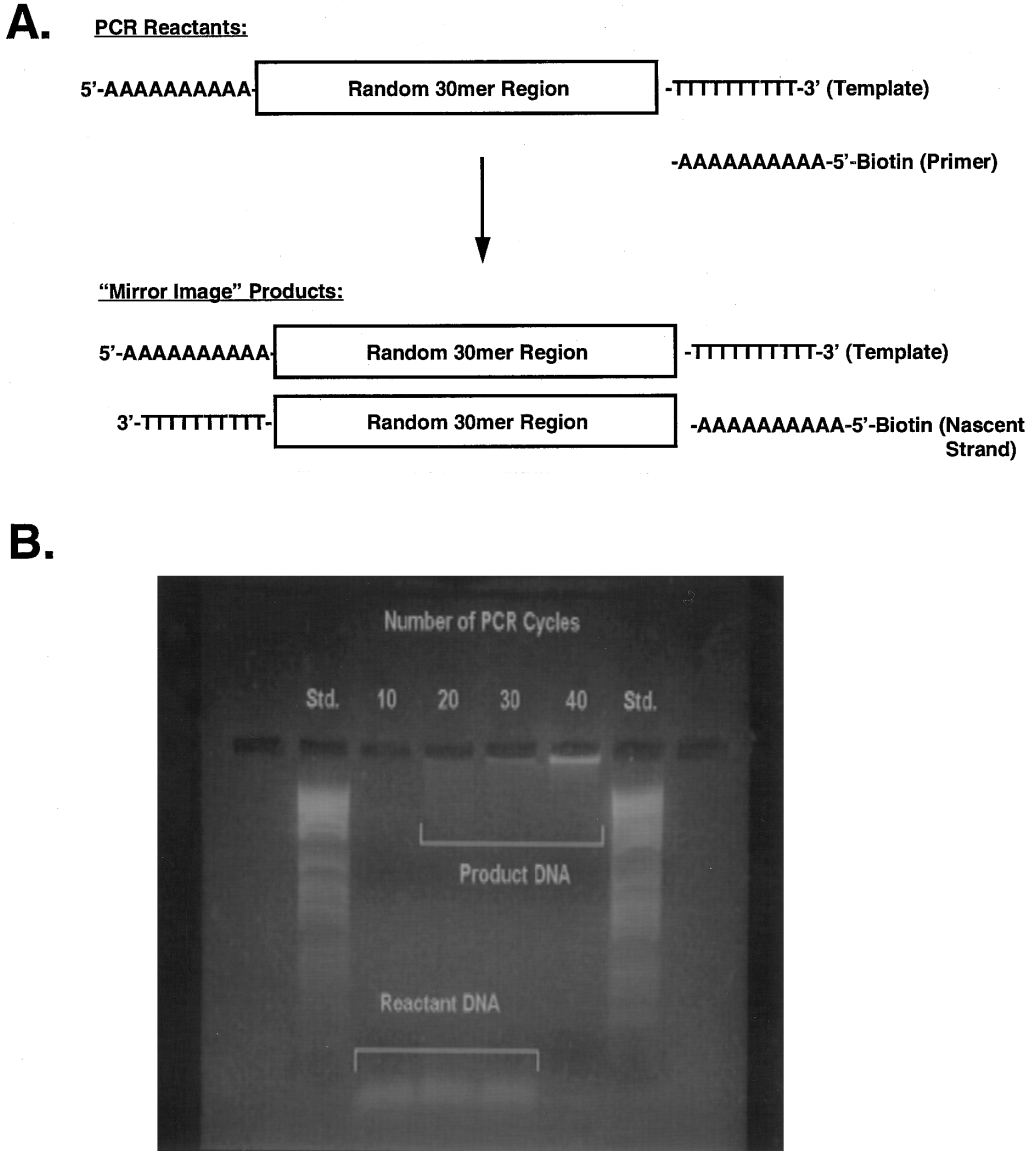

FIG. 2. (A) Simplified PCR scheme employing only one type of poly A primer, and (B) PCR results as a function of PCR cycle showing oddly long (>2.6 kb) PCR products flanked by DNA standards (pGEM; 36-2,645 bp).

fied PCR amplification scheme requiring only one type of primer, and a rapid fluorescence microscopic method for qualitative assessment of nucleic acid binding to antiligand-conjugated MBs following each round of SELEX.

MATERIALS AND METHODS

Chloroaromatic-MB immobilization chemistry. The target analytes; 4-chloroaniline (4-CA), 2,4,6-trichloroaniline (TCA) and pentachlorophenol (PCP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Long chain alkylamine magnetic porous glass (LCA-MPG) MBs were obtained from CPG, Inc., (Lincoln Park, NJ). Tosyl-activated and uncoated M450 (4.5 μm diameter) MBs were obtained from Dynal Corp. (Lake Success, NY). Immobilization was carried out according to each manufacturer's instructions for protein conjugation to MBs except that 1 mg/ml 4-CA, TCA, or PCP in 2-propanol was used.

MB-based SELEX. A 50-mer DNA oligonucleotide library consisting of 5′-poly A 10-mer-randomized 30-mer-3′-poly T 10-mer template sequences and 5′-biotinylated-poly A 10-mer primer (Fig. 2A) were synthesized by Ransom Hill Biosciences, Inc. (Ramona, CA). Random library template (0.5 ng) was amplified with 2 U *Taq* polymerase (Fisher Scientific, Pittsburgh, PA) and 1 μM biotinylated primer (Fig. 2A) using a "hot start" technique. Amplification was performed on a Perkin-Elmer Model 2400 thermal cycler using an initial 5 min (94°C) denaturation followed by 40 cycles of 94°C for 30 sec, 37°C for 30 sec and 72°C for 30 sec, followed by a final 72°C extension step for 7 min. PCR reaction volume was 100 μl. PCR products (7 μl) were electrophoresed in 2% agarose (10 V/cm in cold 1× Tris-Borate EDTA buffer) and

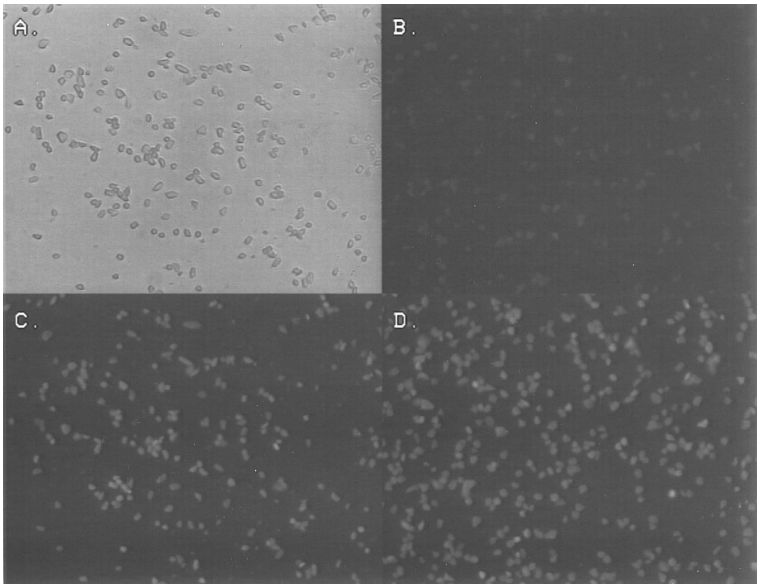

FIG. 3. Fluorescence photomicrographs illustrating (A) the brightfield appearance of LCA-MPG MBs, (B) typical fluorescence microscopic appearance of DNA-4-CA-conjugated LCA-MPG MBs following round 1 of SELEX or any of the controls employed (i.e., background fluorescence) following staining with avidin-FITC (C) brighter appearance of DNA-4-CA-LCA-MPG MBs following round 2 and (D) round 3 of SELEX. Total magnification was 200× for all frames.

stained with ethidium bromide. A ds DNA ladder (pGEM; 36 bp to 2,645 bp range; Promega, Madison, WI) size standard was used. Twenty-five μl of PCR product was added to 75 μl (approx. 4×10^7 MBs) 4-CA-, TCA-, or PCP-conjugated Dynal (tosyl-) and CPG (LCA-MPG) MBs in sterile 1.5 ml microfuge tubes. One hundred μl of 2× binding buffer (2× BB; 1 M NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.5-7.6, (5-7)) was added per tube. Tubes were gently agitated for 2 hr at room temperature (RT). DNA-chloroaromatic-conjugated MBs were washed five times in 1 ml 1× BB using a magnetic separator (CPG, Inc.) to remove unbound DNA and resuspended in 100 μl 1× BB. DNA bound to chloroaromatic-conjugated MBs was released by heating at 94°C for 5 min. Twenty-five μl of heated supernatant containing previously MB-bound DNA was used for the next round of SELEX. This process was repeated three times.

Fluorescence microscopic MB assessment. Twenty-five μl (approx. 10^7) 1× BB washed uncoated M450, tosyl M450, LCA-MPG, and 4-CA-, TCA, and PCP-conjugated tosyl and LCA-MPG MBs (controls lacking bound DNA) and their counterparts with bound DNA from each of three consecutive rounds of SELEX were added to microfuge tubes. Sephadex G25 purified avidin-FITC (Sigma Chemical Co., St. Louis, MO) was diluted 1:1,000 in 1× BB and 200 μl were added to all tubes for 30 min at RT with gentle agitation. MBs were then washed three times in 1 ml 1× BB using magnetic separation. Bead surface fluorescence was assessed using a Nikon Labophot epifluorescence microscope with 100 W Hg arc lamp, 400-440 nm excitation filter, 515 nm dichroic mirror, and 520 nm high pass barrier filter. Images were acquired rapidly to prohibit photobleaching effects with an Optronics (Goleta, CA) DEI-470 CCD color video camera using a $\frac{1}{4}$ sec exposure and output to a Mitsubishi video printer.

RESULTS

PCR results. To test the simplified amplification scheme (i.e., employing only one type of free poly A primer), the random oligonucleotide library was PCR amplified for 10, 20, 30 and 40 cycles. Fig. 2B demonstrates successful amplification of the random library as a function of PCR cycles. Curiously, however, very long PCR products (>2.6 kb) were obtained from the 50-mer template. This may be due to partially hybridized random regions that concatenate to form a much larger product or triple helical DNA formation. Investigation of this phenomenon is the subject of future research. It was, however, apparent that a PCR product was generated using the random template and single primer as the accumulation of product increased exponentially with PCR cycles. The low annealing temperature (37°C) was necessary to allow for formation of a PCR product, since annealing temperatures >40°C gave no product (data not shown). Unfortunately, the low annealing temperature may have also encouraged formation of the long amplification product.

Fluorescence microscopic assessment. Fig. 3 illustrates the typical increase in fluorescence intensity of 4-CA-conjugated LCA-MPG MBs as a function of SELEX rounds. Conjugated MBs collected after the first round of SELEX (Fig. 3B) demonstrated little, if any, fluorescence above background from uncoated and variously conjugated control MBs lacking exposure to the DNA library. However, similar MBs gleaned following rounds 2 and 3 (Figs. 3C and 3D, respectively) of the SELEX process showed marked increases in numbers of fluorescent MBs and overall

fluorescence intensity. Similar results were obtained from TCA and PCP SELEX experiments (data not shown).

DISCUSSION

The present work describes several novel approaches to development of DNA ligands to chloroaromatics by SELEX. Use of MBs was a novel and advantageous approach to affinity separation as it obviated elution from an affinity column with excess analyte and eliminated the need for precipitation. Instead, DNA bound to the chloroaromatic-conjugated MBs could be directly used as the source of template in PCR amplification following affinity separation.

A simplified PCR scheme, requiring only one type of primer, was shown to successfully amplify the DNA library. The nascent strand ends are "mirror images" of the original template ends (Fig. 2A). Thus, the 5′-biotinylated poly A 10-mer can also primer the 3′-poly T end of the nascent strand to propagate the PCR. Although, a curiously large PCR product was obtained, this is virtually irrelevant in terms of oligonucleotide binding to conjugated MBs.

Results of fluorescence microscopic binding assays indicated that significant levels of higher affinity DNA were binding to chloroaromatic-conjugated LCA-MPG MBs following round 2 of SELEX. Tosyl surface chemistry proved unsuccessful for chloroaromatic SELEX. Thus, the 15 Å linker arm on LCA-MPG MBs appears necessary for successful SELEX. Also of interest is that Ellington and Szostak (6) found ss DNA library subsets bound efficiently to organic dyes, while ds DNAs did not, thus raising the possibility of a ss region or other phenomenon to account for the empirical fluorescence results seen here.

The present work extends SELEX technology by demonstrating applicability to important environmental pollutants (chloroaromatics). Considering the relatively high cost, laborious nature and low efficiency associated with hapten immunization for both polyclonal and monoclonal antibody generation, SELEX seems an attractive alternative.

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