In vitro cloning of complex mixtures of DNA on microbeads: Physical separation of differentially expressed cDNAs

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Contributed by Sydney Brenner, December 14, 1999

We describe a method for cloning nucleic acid molecules onto the surfaces of 5-μm microbeads rather than in biological hosts. A unique tag sequence is attached to each molecule, and the tagged library is amplified. Unique tagging of the molecules is achieved by sampling a small fraction (1%) of a very large repertoire of tag sequences. The resulting library is hybridized to microbeads that each carry $\approx 10^6$ strands complementary to one of the tags. About 10^5 copies of each molecule are collected on each microbead. Because such clones are segregated on microbeads, they can be operated on simultaneously and then assayed separately. To demonstrate the utility of this approach, we show how to label and extract microbeads bearing clones differentially expressed between two libraries by using a fluorescence-activated cell sorter (FACS). Because no prior information about the cloned molecules is required, this process is obviously useful where sequence databases are incomplete or nonexistent. More importantly, the process also permits the isolation of clones that are expressed only in given tissues or that are differentially expressed between normal and diseased states. Such clones then may be spotted on much more cost-effective, tissue- or disease-directed, low-density planar microarrays.

DNA analysis | gene expression | parallel cloning | fluid microarray

Analysis of complex genomes requires methods for generating and fractionating many tens of thousands of DNA fragments in quantities and formats amenable to biochemical analysis. The difficulty of such analysis is illustrated by human gene expression: the human genome is estimated to contain about 100,000 genes, of which 10–30%, or about 20–40 Mb, are actively expressed in any given tissue (1–2). Such large numbers of expressed genes make it difficult to track changes in expression patterns, particularly in view of the large fraction of genes that are expressed at very low levels: it has been estimated that as much as 30% of mammalian messenger RNA consists of many thousands of distinct species each making up far less than a few tenths of a percent of the total, and typically averaging less than a few tens of copies per cell (3–4). Yet relatively minor alterations in gene expression patterns are associated with profound changes in cell physiology and, more broadly, in the state of an organism's health, its longevity, and its survival (5–7). A variety of techniques has been developed for analyzing gene expression that differ widely in convenience, expense, and sensitivity. Presently, techniques based on direct sequence analysis or specific hybridization of complex polynucleotide probes to microarrays of oligonucleotides or polynucleotides provide the most comprehensive and sensitive analysis of gene expression (8–9). However, in both approaches, the sequences to be analyzed must either be known or cloned and processed individually beforehand, usually with the aid of complex robotics systems. This makes it difficult to isolate and/or monitor many potentially important genes that are differentially expressed at low absolute levels against a background of more abundantly expressed genes.

To address some of these problems, we describe an approach that greatly simplifies the handling and analysis of complex mixtures of cDNA or genomic fragments. We show how millions of nucleic acid molecules, amplified with one set of common primers, can be cloned and specifically attached to 5-μm microbeads in a few single-tube reactions. Central to the method is the formation of a repertoire of oligonucleotide tags assembled combinatorially from a defined set of subunits, or "words," and their attachment to individual polynucleotides of a complex mixture. By making the repertoire of tags large relative to the number of polynucleotides in the mixture, samples of the tag–polynucleotide conjugates may be selected with virtually every polynucleotide having a unique tag. Samples of conjugates then may be amplified and specifically hybridized to their complementary sequences (anti-tags) on separate microbeads in a single reaction to form a library of microbeads, each having attached a clonal population of one polynucleotide from the original mixture.

The key advantage of this approach over biological cloning is that the DNA on the surface of each microbead is readily accessible for biochemical analysis without further processing. In addition, all clones in a microbead library can be interrogated simultaneously by analytical probes designed to assay specific properties. This enables a broad range of applications in which clones are identified or physically fractionated by virtue of their sequence or their abundance or their ability to bind particular ligands. Below, we show how such "microbead clones" can be used to extract differentially regulated genes, independently of any sequence information, in the human acute monocytic leukemia cell line, THP-1, induced by phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS). After competitive hybridization of cDNA probes from the induced and noninduced cells, microbeads sorted on the basis of fluorescence intensity ratios were found to carry sequences known to be up-regulated and down-regulated in THP-1 cells after induction by PMA and LPS. Further validation is provided by plaque hybridization with probes constructed from several genes fractionated by our microbead procedure.

Materials and Methods

Construction of Oligonucleotide Tag and Anti-Tag Libraries. A library of 32-mer tags was synthesized based on eight 4-mer "words"

Abbreviations: PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorting; FAM, 6-carboxyl-fluorescein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AW059512–AW059953).

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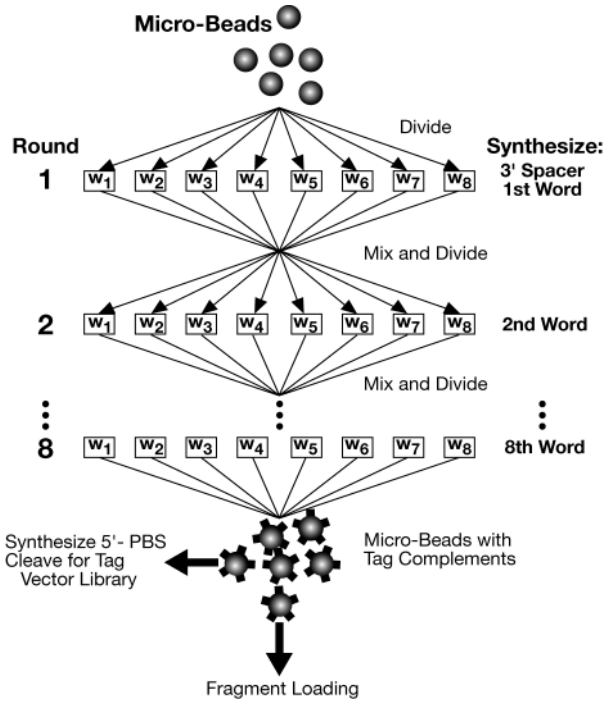

Fig. 1. Tags were synthesized by eight rounds of combinatorial synthesis, wherein each word, w_1–w_8, is added in a separate column of a DNA synthesizer. In each round, a word was added to each growing tag by four conventional base-coupling cycles, after which the microbeads were mixed and divided for the next addition. After the eighth round, a portion of the microbeads were separated for further synthesis of a 5′ primer-binding site (PBS), followed by cleavage, amplification, and insertion into pLCV1.

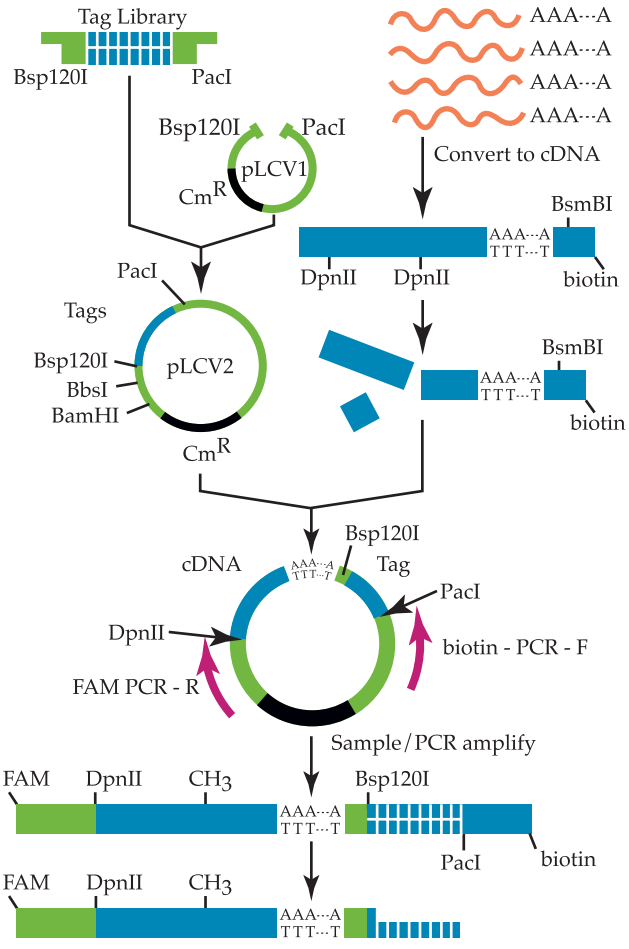

Fig. 2. Attachment of tags to cDNAs.

lacking dG described in *Results* by eight rounds of "mix and divide" combinatorial synthesis (Fig. 1) on 40 billion 5-μm glycidal methacrylate microbeads (Bangs Laboratories, Carmel, IN). The microbeads were prepared with ≈10% of the initial nucleotides linked by a base-labile group (5′-phosphate-ON, CLONTECH). A 28-mer 3′ spacer having a primer-binding site and a *Pac*I site was synthesized, followed by synthesis of the 32-mer tags and three cytidylate residues. Five billion of these microbeads were removed and by further synthesis a *Bsp*120I site and a 5′ primer-binding site were added. Aliquots of the remaining 35 billion microbeads were prepared for capturing tagged cDNAs as follows: 2.5×10^8 microbeads suspended in 100 μl of H_2O were combined with 100 μl of 10× NEB buffer 2 (New England Biolabs), 10 μl of 100 mM ATP, 1 μl of 10% Tween 20, 17 μl of T4 polynucleotide kinase (10 units/μl), and 772 of μl H_2O for a final volume of 1,000 μl. After incubating for 2 hr at 37°C with vortexing, the temperature was increased to 65°C for 20 min to inactivate the kinase, with continued vortexing. After incubation, the microbeads were washed twice by spinning down the microbeads and resuspending them in 1 ml of Tris-EDTA containing 0.01% Tween 20. Postsynthesis analysis of these showed that each microbead carries about 10 million copies of a given anti-tag sequence (data not shown).

The aliquot of five billion microbeads was treated to cleave DNA attached by base-labile linkers to yield a total of 1×10^{16} sequences. After deprotection and purification by reverse-phase chromatography, sequences were isolated by ethanol precipitation, resuspended, and used as the template for a T7 polymerase fill-in reaction with a primer complementary to the 3′ primer-binding site. The entire duplex then was PCR-amplified with primers specific for the 3′ and 5′ primer-binding sites, after which the amplicon was digested with *Pac*I and *Bsp*120I and inserted into pLCV1 (Fig. 2). The product was electroporated into *Escherichia coli* to produce more than 200 million independent chloramphenicol-resistant clones, designated pLCV2.

cDNA Synthesis and Attachment of Tags. mRNA was converted to cDNA, tagged, and loaded onto microbeads as described (10). Briefly, poly(A)$^+$ RNA was extracted by using a FastTrack 2.0 kit (Invitrogen), using the manufacturer's protocol. Double-stranded cDNA was synthesized by using a cDNA Synthesis kit (Stratagene), using the manufacturer's protocol with the following modifications: 2.5 μg of mRNA was used for each synthesis and the primer for first strand synthesis was 5′-biotin-GACATGCTCGTCTCTGCAT$_{19}$V. After second-strand synthesis, the cDNA was size-fractionated on a SizeSep 400 column (Amersham Pharmacia) and ethanol-precipitated. The cDNA was resuspended and digested with 100 units of *Dpn*II at 37°C for 2 hr, after which the biotinylated cDNA was purified with Dynal M-280 streptavidin beads. cDNA fragments were digested off the beads by using 40 units of *Bsm*BI for 2 hr in an Eppendorf Thermomixer set at medium speed at 37°C. The supernatant was collected, and the beads were resuspended for a second *Bsm*BI digestion. cDNA fragments from the first and second digestions were pooled, ethanol-precipitated, and resuspended in 10 μl of H_20. Tag vector pLCV2 was used for loading libraries on microbeads. pECV, which lacks tags, was used for probe libraries. The vectors were digested with *Bbs*I and *Bam*HI and dephosphorylated, and aliquots of each cDNA preparation were ligated with each vector. Sequences adjacent to *Bbs*I and *Bsm*BI were chosen to ensure compatible ends. DNA was transformed

into electro-competent *E. coli* TOP10 cells (Invitrogen). Aliquots were titered on LB agar plates containing 30 μg/ml chloramphenicol, and library pools were grown in liquid cultures. For the microbead libraries, six pools of 160,000 clones each were grown in 50-ml liquid cultures. For the probe libraries, 1×10^7 clones were grown in 1-liter cultures. Plasmid DNA was prepared and used for subsequent manipulations.

Microbead Loading. Tagged cDNAs (160,000) were amplified in the presence of 5-methyl dCTP using flanking PCR primers (designated biotin-PCR-F and FAM (6-carboxyl-fluorescein)-PCR-R; labeling reagents from CLONTECH), and the products were digested to completion with *Pac*I and affinity-purified. To expose the tags as single strands, amplified DNA was treated with T4 polymerase in the presence of 1 mM dGTP for 60 min, thereby digesting a single strand of the tag to the GC-rich *Bsp*120I site. The reaction was stopped with EDTA, and the enzyme was denatured by heating (72°C for 15 min). Fifty micrograms of this mixture then was combined with an aliquot of 16.7 million microbeads, each having about 10^6 copies of a single anti-tag, in a 100-μl reaction containing 500 mM NaCl, 10 mM sodium phosphate, 0.01% Tween 20, and 3% dextran sulfate. The sample was incubated for 3 days at 72°C. The microbeads then were washed twice, first in 50 mM Tris, 50 mM NaCl, 3 mM Mg_2Cl, and then in 10 mM Tris·HCl (pH 8), 1 mM EDTA, 0.01% Tween 20, after which the 1% brightest beads were sorted on a Cytomation MoFlo cytometer. cDNAs (10^4-10^5 per microbead) were loaded, determined by comparing the amount of test probe hybridized to saturation to 2,000 identical microbeads with known amounts of the test probe after elution and electrophoretic separation (data not shown). Loaded, sorted microbeads were treated with T4 DNA polymerase in the presence of 0.1 mM dNTP for 30 min at 12°C to fill in any gaps between the hybridized conjugate and the 5′ end of the anti-tag. The reaction was stopped by washing in buffer containing 10 mM Tris·HCl (pH 8), 1 mM EDTA, and 0.01% Tween 20, after which the microbeads were treated with Pronase (Boehringer Mannheim) at a final concentration of 0.14 μg/μl in PBS containing 0.01% Tween 20, and incubated for 60 min at 37°C. The anti-tag was ligated to the cDNA by treating with T4 DNA ligase in the presence of 1 mM ATP for 60 min at 37°C. After washing and Pronase treatment, the fluorescent label was switched to the noncovalently attached strand by digesting the DNA with *Dpn*II, treating with Pronase, treating with phosphatase, and ligating an adaptor carrying a 3′ FAM label. A 14-bp adaptor having a phosphorylated 5′ *Dpn*II-compatible end and a 3′-FAM label was ligated in a conventional ligation reaction, after which the microbeads were washed once with PBS containing 1 mM $CaCl_2$ and treated with Pronase. The remaining nick was ligated by treatment with T4 polynucleotide kinase and T4 DNA ligase, after which the microbeads were washed once with PBS containing 1 mM $CaCl_2$, treated with Pronase, and washed twice with Tris-EDTA/Tween. Noncovalently attached strands were removed by treatment with 150 mM NaOH.

Probe Synthesis and Competitive Hybridization. cDNA-containing plasmid libraries from induced and noninduced THP-1 cells were linearized, and probes were prepared by runoff synthesis. Twenty micrograms each of the probe libraries was digested with *Sau*3A. Thirty-five rounds of linear amplification with either R110 dUTP (Perkin–Elmer) or Cy5 dUTP (Amersham Pharmacia) were performed. The strands complementary to the DNA covalently attached to reference microbeads (described below) were melted off with two 0.5 ml of 150 mM NaOH washes at room temperature for 15 min with mild vortexing, after which the microbeads were washed twice in 0.5 ml of 4× SSC 0.1% SDS. Ten micrograms of each probe then was mixed with the microbeads in 50 μl of 4× SSC 0.1% SDS, heated to 80°C for 3 min, and then cooled to 65°C. After 16 hr with constant mixing, the microbeads were quenched in 10 ml of Tris-EDTA/Tween on ice, rinsed with 1× SSC/0.1% SDS, resuspended in 0.5 ml of 1× SSC/0.1%SDS, and finally washed in 0.1× SSC/0.1% SDS at 65°C for 15 min. Labeled microbeads were sorted with a Coulter EPICS Elite ESP flow cytometer.

PCR, Cloning, and Sequencing of Isolated cDNA. Sorted microbeads were used directly in a PCR, and the product was cloned by using the TA Cloning procedure (Invitrogen) and sequenced.

Cell Culture. THP-1 cells (ATCC accession no. TIB-202) were grown in DMEM/F-12 media supplemented with 10% heat-inactivated FBS (56°C, 30 min), 5×10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures seeded with 1×10^3 cells per ml were grown to a cell density of 1×10^6 cells per ml (approximately 4 days). Cells were induced by adding PMA (stock concentration 1 mM in DMSO) to a final concentration of 100 nM and were grown for 48 hr with PMA. DMSO was added to noninduced cells. During this period, approximately 80% of the PMA-treated cells became adherent, whereas about 20% remained in suspension. After 48 hr, the medium and nonadherent cells were removed from PMA-induced cells and replaced with fresh medium containing 5 μg/ml LPS from *E. coli* serotype 0111:B4 (Sigma). Control cells were pelleted by centrifugation at 1,400 × *g* for 4 min and resuspended in fresh equilibrated medium, and incubation continued. The cells were harvested after 4 hr. Adherent PMA- and LPS-treated cells were dislodged by using a cell scraper and pelleted by centrifugation at 1,400 × *g*. Control cells, which remained in suspension, were simply pelleted.

Results

Principle of the Method. Each nucleic acid molecule in a complex mixture first is labeled with an oligonucleotide tag, after which a sample of the resulting library of tag–molecule conjugates is amplified. After amplification, a sample of *n* molecules will yield a library containing *n* families of clones, each identified by a unique tag. This permits them to be collected on a microbead that carries the complement (anti-tag) to a family's unique tag. Thus, a mixture of 1,000,000 molecules can be converted into a library of about as many microbeads, each carrying about 100,000 copies of one of the templates. To achieve this, the nucleic acid sequences used as tags and anti-tags must be chosen to maximize the efficiency and discrimination of hybridization of a given clone to a given microbead.

Six criteria were used to design the repertoire of tags: (*i*) the repertoire must be diverse enough to enable the unique tagging of all (or nearly all) the molecules in large libraries; (*ii*) the tags must remain physically attached to the tagged molecules after operations such as cleavage by restriction enzymes; (*iii*) the melting temperatures (T_m) of all tag/anti-tag duplexes must be isothermal; (*iv*) the difference in T_m between any perfectly matched tag/anti-tag duplex and any duplex with a single mismatch must be both the same for all sequences and large enough to discriminate strongly in favor of the perfect match; (*v*) a practical and effective manner must exist to enable construction of such a repertoire; and (*vi*) there must be a simple way of applying the method, in parallel, and in one reaction, to any ensemble of nucleic acid molecules. A library of sequences with these properties can be constructed from a special DNA language with a vocabulary made up of eight four-base "words": TTAC, AATC, TACT, ATCA, ACAT, TCTA, CTTT, and CAAA.

Each word uses only three (A, T, and C) of the DNA bases and differs from all of the other words in three of the four bases, and they all form, with their respective complements, three A:T and one G:C base pairs. Limiting the composition of the four-

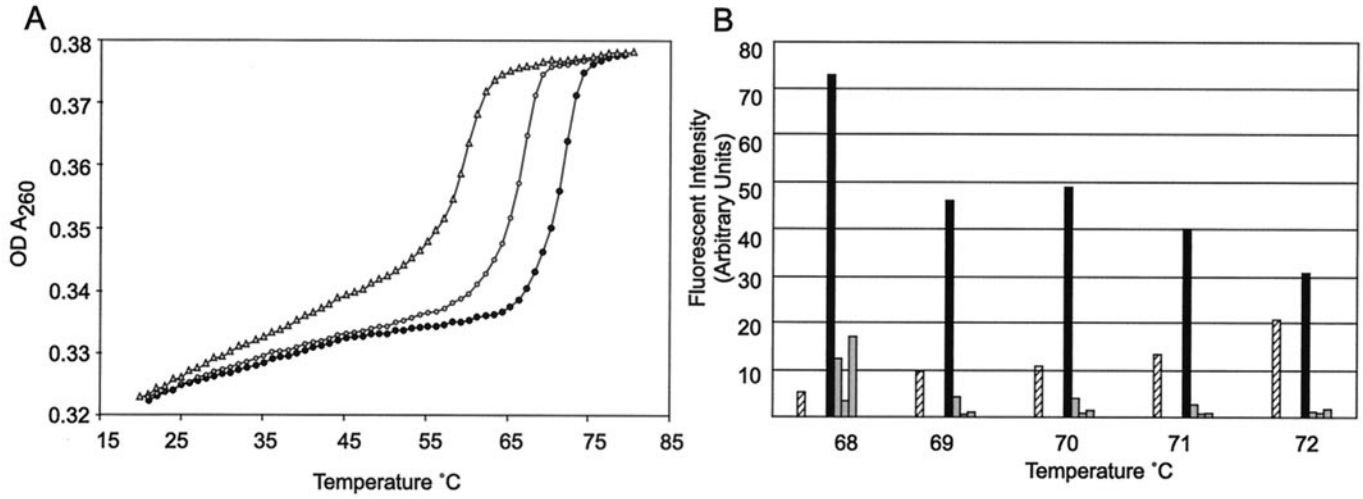

Fig. 3. Melting curve of tags with complements. (*A*) Approximately 0.35 OD units of each double-stranded oligonucleotide was resuspended in 10 mM sodium phosphate, pH 7.6/50 mM NaCl/3 mM $MgCl_2$. The samples were heated at 1°C per min, and the OD at 260 nm was measured and recorded. Reactions consisted of one common oligonucleotide, cccatcactttatcaatcaacatatcacaaaaatctcc, and a second oligonucleotide as follows: perfect match, (●) ggagatttttgtgatatgttgattgataaagtgatggg; one-word mismatch, (○) gg*atgat*tttgtgatatgttgattgataaagtgatggg; two-word mismatch, (△) ggagatttttgt*ttga*gtt*ttgt*gataaagtgatggg. (*B*) Microbeads bearing the oligonucleotide ggagatttttgtgatatgttgattgataaagtgatggg were loaded with FAM-labeled cDNA tagged with this sequence. In separate tubes, three cDNAs whose tags differed at the first, third, or fifth positions also were loaded. The samples then were washed in 50 mM Tris/50 mM NaCl/3 mM Mg_2Cl at increasing temperatures as indicated. Microbeads then were analyzed by FACS, and the means of fluorescence intensities were plotted for the perfect match of the tag (filled bar), the mismatches (gray bars), and the ratios (cross-hatched bar) of the perfect signal to the average noise of the three mismatches.

nucleotide words to three bases eliminates self-complementarity within any sequence made up of the words and prevents their cleavage by any restriction enzyme with symmetrical recognition sites containing both G and C. The repertoire of tags is constructed by a "mix and divide" combinatorial synthesis of all possible eight-word combinations. There are 8^8 such combinations, forming a tag repertoire of 16,777,216 sequences 32 bases long. An example of one tag and its anti-tag complement is shown below:

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5′-TACT.TTAC.ACAT.ATCA.CTTT.CTTT.CAAA.AATC-3′
3′-ATGA.AATG.TGTA.TAGT.GAAA.GAAA.GTTT.TTAG-5′
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Because of the words used to construct the tag repertoire, all 16,777,216 eight-word tag/anti-tag duplexes have the same base pair composition, i.e., 24 A:T and eight G:C base pairs. Consequently, all of the tags in the entire repertoire should have about the same T_m. Because all tag sequences differ from their nearest neighbors by at least one word, or three base mismatches, there is good discrimination between perfect matches and one-word mismatches. Fig. 3*A* depicts the melting curves of eight-word duplexes that have zero-, one-, or two-word mismatches. It shows that at 68°C, a duplex with a single-word mismatch dissociates, whereas the duplex with an exact match does not. Fig. 3*B* shows that anti-tags immobilized on a solid surface do not lose their ability to discriminate in favor of the correct tags in hybridization experiments. To estimate the accuracy of loading, we deposited individual microbeads into 96-well microtiter plates by using fluorescence-activated cell sorting (FACS) and subjected them to multiple rounds of PCR amplification. Ten percent of the wells produced no detectable product, 70% yielded single bands on an agarose gel, and the remaining 20% yielded more than one band (data not shown). Of this latter category, one-fourth were estimated to have contained two microbeads; we estimate, therefore, that at least 85% of the microbeads contain only one sequence.

Allocation of tags to individual molecules is outlined in Fig. 2. First, the complete repertoire of tags in a plasmid library is ligated to the entire population of cDNAs to give a population of tag–cDNA conjugates that contains a conjugate between every tag and every cDNA. Next, a sample of 160,000 tag–cDNA conjugates is taken that includes only 1% of the full repertoire and, thus, the probability that two different cDNAs will have the same tag sequence is very small, about 0.01%. The vast majority will be uniquely tagged. The small sample size also ensures greater specificity in hybridization, because most of the sampled tags are likely to differ from one another by two or more words. The 160,000 tag–cDNA conjugates are amplified by PCR to give fluorescently labeled biotinylated amplicons. After purification with a streptavidinated support, the DNA is treated with T4 DNA polymerase in the presence of dGTP to remove one strand from the tag/anti-tag duplex using the $3' \rightarrow 5'$ exonuclease and exchange reaction activities of the enzyme. The resulting tag–cDNA conjugates are loaded onto microbeads by mixing them with the full 16.7 million repertoire of microbeads, each carrying a specific anti-tag sequence. The loading reaction is carried out under equilibrium conditions (i.e., 72 hr at 72°C) to ensure that hybridization discrimination is achieved through the higher off-rate of mismatched sequences. After separating loaded microbeads from unloaded microbeads by FACS, as shown in Fig. 4, the hybridized DNA is ligated to the anti-tag, covalently attaching one strand of the DNA to the microbead's surface. This permits easy removal of the noncovalently attached strand.

Analysis of Differential Gene Expression. To illustrate the first of several applications of our cloning methodology, we describe an experiment for physically extracting clones that are differentially represented in two libraries. After melting off the noncovalently attached DNA strands from the microbeads, the remaining strands are available for competitive hybridization with two probes labeled with different fluorophores, to produce, in effect, a fluid microarray of millions of microbeads. The ratio of probes hybridizing to each bead can easily be measured at high throughput (20,000 beads per sec) by using available FACS equipment. Thus, microbeads labeled with more of one fluorophore than the other can be easily identified and physically separated from the rest of the library.

The sensitivity of this approach was tested in an experiment where two fluorescent probes in ratios of 1:8, 8:1, 1:4, 4:1, 1:2,

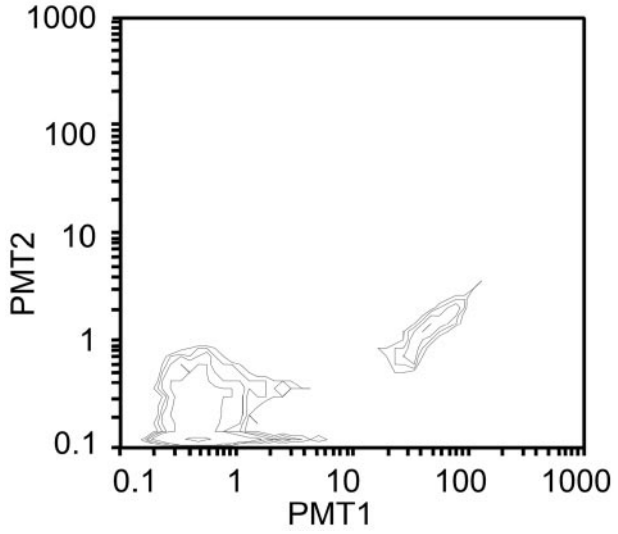

Fig. 4. Two-parameter, 530-nm (PMT1) and 666-nm (PMT2), FACS contour plot showing separation of loaded microbeads (contour on right) from unloaded microbeads (contour on left).

2:1, 1:1, 0:1, and 1:0 were hybridized to a complementary set of microbeads. The results (Fig. 5) show that the probe ratios can be clearly measured and that differences as low as 2-fold can be detected. The range of fluorescence intensities exhibited by the microbeads reflects the variation in the number of cDNAs loaded on each microbead, which determines the total number of probe molecules that can bind.

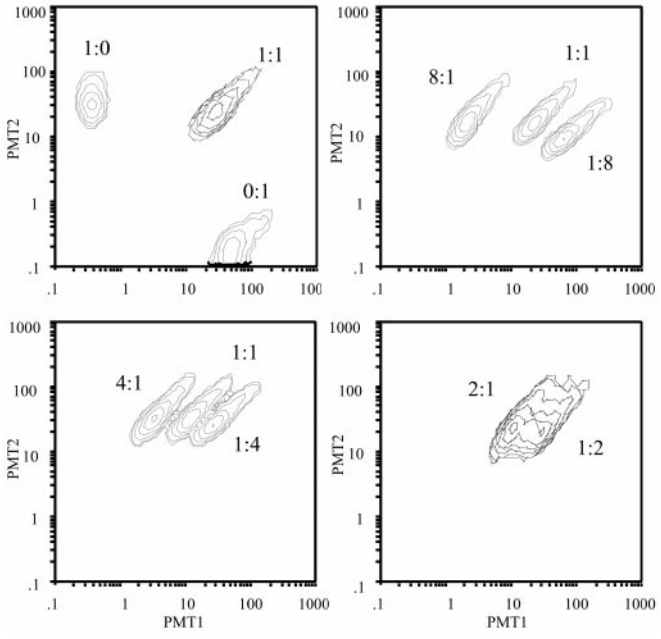

Fig. 5. Model system for two-color competitive hybridization. A 34-mer, ggagatttgataaagtttgatgtgtaatagaggg, was synthesized with a 3′ FAM or Cy5 label. The oligonucleotides were brought to final concentration of 14 μM in 30 μl. The two labeled oligonucleotides were combined in nine separate mixtures in ratios of 1:0, 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8, and 0:1. Hybridization of each mixture to 100,000 microbeads bearing the complementary oligonucleotide was performed, and 10,000 microbeads from each of the mixtures were analyzed with a Coulter Elite Flow Cytometer using 488-nm and 633-nm lasers.

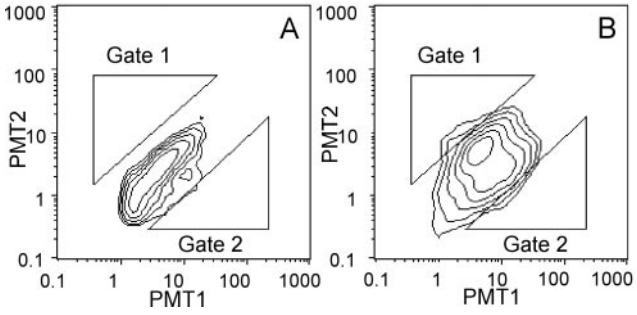

Fig. 6. A reference library of 2,000,000 microbeads was formed by mixing equal numbers of microbeads with attached cDNA derived from induced and noninduced THP-1 cells. Reference microbeads (100,000) were hybridized with 10 μg each of Cy5-labeled probe and R110-labeled probe, both derived from the same induced library to yield *A*. Microbeads (1,600,000) were hybridized with 10 μg of Cy5-labeled probe from the induced library and 10 μg of R110-labeled probe derived from the noninduced library to yield *B*.

To see whether we could enrich for differentially expressed genes by using FACS, we made a microbead library with cDNA pooled from THP-1 before and after treatment with PMA and LPS. Four cDNA probes were constructed: two from the same library of induced cDNAs, which were labeled with either R110 or Cy5, and one each from libraries of induced and noninduced cDNAs, which were labeled, respectively, with Cy5 and R110. The former were used in a control experiment to compare the library with itself. Fig. 6*A* is a FACS plot of the microbead library hybridized with a 1:1 mixture of differently labeled probes from the same library, whereas Fig. 6*B* is a plot of the microbead library hybridized with a 1:1 mixture of cDNA probes from the induced and noninduced cells. The distribution of microbeads in Fig. 6*A* allowed us to set gates for collecting microbeads that were more heavily labeled with either Cy5 or R110 (Fig. 6*B*). The triangles in Fig. 6*B* represent the gates from which microbeads carrying up-regulated or down-regulated clones were collected. Each gate was set to collect about 1% of the total number of clones in the library: gate 1 should collect clones that are 10-fold or more up-regulated, whereas gate 2 should collect clones that are 2-fold or more down-regulated.

Of the 1,600,100 microbeads used in this experiment, 13,988 (0.87%) were collected in the up-regulated fraction, and 17,303 (1.01%) in the down-regulated fraction. The DNA in these fractions was recovered by PCR, and 956 of the up-regulated clones and 985 of the down-regulated clones were sequenced. Clones from the up-regulated fraction included 68 known genes, 35 expressed sequence tags, and nine novel sequences, whereas clones from the down-regulated fraction included 209 known genes, 111 expressed sequence tags, and five novel sequences. In the up-regulated fraction there were many known PMA-induced genes such as IL-8, tumor necrosis factor α, macrophage inflammatory protein 1, and superoxide dismutase 2 (11–14). There was very little overlap between the up- and down-regulated sets of genes, except for the presence of a small number of microbeads bearing up-regulated B94 (M92357) cDNAs in the down-regulated fraction and some microbeads bearing down-regulated 23 kDa basic protein transcript (X56932) in the up-regulated fraction. These were likely the result of sorting errors caused by clustering of microbeads in adjacent drops.

Plaque Hybridizations. To validate our procedure, we independently cloned cDNA from the induced and noninduced THP-1 cells in bacteriophage lambda and screened filters each containing 50,000 plaques with probes derived from 19 of the genes identified by sequencing the DNA from the fractionated beads. Table 1 summarizes these results. In every case, the sequences

GENETICS

Table 1. THP-1 expression levels by plaque hybridization assays

GeneBank Number	Gene	Noninduced, replicate counts	PMA/LPS induced, replicate counts	Up- or down-regulation
Up-regulated genes (Gate 1)				
Y00787	IL-8	1/0	663/808	>500×
X65965	SOD-2	0/0	1636/1096	>500×
M25315	MIP-1	0/0	1120	>500×
J04130	Act-2	0/0	453/542/572/606	>500×
X02910	TNF-α	0/0	81/82	>500×
J03210	Collagenase type IV	0/0	92/93	>500×
M21121	RANTES	0/7	113/92	29×
M92357	B94	7/3/0/2	155/150/124/174	50×
Down-regulated genes (Gate 2)				
X13546	HMG-17	75/83	6/4	15.8×
M17885	Acidic ribosomal protein P0	191/195	84/54	2.8×
X16869	Elongation factor 1α	577/607	268/270	2.2×

derived from beads collected in gates 1 or 2 were shown to be either up-regulated 29- to 500-fold or down-regulated 2- to 16-fold, respectively. These changes spanned expression levels of 0.02–2%.

THP-1 cells respond to PMA by first coming out of the cell cycle followed by the induction of the genes required for differentiation. The first process should be associated with the down-regulation of a large number of genes involved in the growth of cells, such as ribosomal protein-coding genes. Plaque hybridization with several genes in this class indicates that these are down-regulated between 2- and 4-fold (Table 1). Our method detects these relatively small changes; we found an enrichment of these cDNAs in the pool of microbeads collected in gate 2. Of the 989 clones sequenced, 375 could be assigned to 48 ribosomal proteins with recurrences ranging from 1 to 42 and an average of 7.8 per gene. We estimate the enrichment to be 5×. In addition, the pool of microbeads in gate 2 was not significantly contaminated by genes that were unaffected by PMA stimulation (data not shown).

Discussion

We have described a method for cloning libraries of DNA fragments on the surfaces of microbeads and have shown how this method facilitates identification and physical extraction of genes that are differentially expressed. Microbead clones can be processed in parallel in a single tube, and there is no need to handle each clone separately for analysis and retrieval. Because the DNA on microbeads is accessible for direct interrogation, no further biochemical processing is required for various types of analyses, such as hybridization or sequencing.

Our ability to generate a million or more microbeads carrying clones from very large cDNA libraries allows the selection of sequences differentially regulated at very low levels. All that is necessary is that the microbead library be large enough to contain nearly all of the sequences from the libraries being compared. No previous knowledge of any of the sequences is required. Nucleic acid samples therefore can be from any source. The microbead libraries used for comparisons are constructed from a combination of the libraries being analyzed. Probes are constructed separately from the libraries and are labeled differentially. The ratio with which probes hybridize to the microbeads is equally accurate for microbeads carrying rare and abundant species, because each microbead responds to the probes independently of how many others carry the same cDNA.

Most importantly, our method allows libraries to be probed very deeply, much more deeply than is practical by other methods. To attempt identification of differentially expressed clones by deep, random, expressed sequence tag sequencing of the libraries being compared would be prohibitive. To do so with high-density planar microarrays (chips or gridded clones on filters) would require access to chips or filters containing all sequences expressed by the system and, even then, might not adequately detect low abundance genes. To do so with serial analysis of gene expression experiments limited to 2,000 sequencing runs (the same number we used) would be equivalent to probing only about 50,000 signatures deep whereas our extracted, differentially expressed, clones were the result of probing the libraries 2,000,000 deep. Because our technique readily selects out a much-reduced set of clones relevant to a biological system or an experiment, it can be deployed as a discovery tool that enables easier and more effective uses of other techniques.

We thank Laurie J. Goodman, Holger Hiemisch, Hans Christian Kornau, An Lai, Ling Yau, Jason Zeng, and Eugene A. Zhukovsky for their assistance in this project.

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