Subtractive hybridization system using single-stranded phagemids with directional inserts

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Received April 16, 1990; Revised and Accepted July 2, 1990

ABSTRACT

We describe a subtractive hybridization protocol which is designed to permit subtractions between cDNA libraries. The method uses single-stranded phagemids with directional inserts as both the driver and the target. We modified the M13 phagemid vector pBluescript for the directional cDNA cloning and subtractive hybridization. Two simplified methods for efficient construction of directional cDNA libraries are also described. Using a model system, we found that one round of subtractive hybridization results in a 5,000-fold specific subtraction of abundant molecules. We used two methods to quantify the efficiency and verify the specificity of the subtraction. In order to obtain these subtraction efficiencies, it was necessary to develop a method to purify the single-stranded DNA to homogeneity. The single-stranded purification involved using potassium iodide (KI) density centrifugation, restriction endonuclease digestion and phenol extraction in the presence of magnesium. We describe the several advantages of using directional inserts for the subtraction procedure.

INTRODUCTION

Subtractive hybridization of nucleic acids is a powerful method to isolate the coding sequences of genes which are differentially expressed, such as during development or in disease states (1, 2, 3, 4, 5, 6, 7). Until recently, subtractive hybridization experiments typically involved hybridizing tens of micrograms of poly A⁺ RNA (driver) with at least ten-fold less cDNA (target) prepared from another source. The RNA-DNA hybrids were separated from the unhybridized cDNA using hydroxylapatite chromatography. The remaining cDNA was either cloned (7) or used as a probe in a differential screening procedure (3). While this method has been useful, it suffers from an important drawback, in that it requires large amounts of driver mRNA. This requirement can be a major obstacle if one needs to prepare the driver mRNA from a difficult to obtain tissue, such as from parts of an embryo. Furthermore, following the

subtraction, typically only a few nanograms of target sequences remain; hence it is difficult to obtain large numbers of clones from this material.

We have developed an improved method of subtractive hybridization which circumvents many of the problems facing the standard protocols. Our method uses single-stranded phagemid vectors as both the driver and target. This approach was first described by Duguid and co-workers (5, 8). However, our method, which was independently conceived, has several important differences; foremost are the directional insertion of cDNAs (or any other DNA fragment) into single-stranded phagemids, the methods we use to quantitate the efficiency of subtraction and the protocol which we employ to purify the single-stranded DNA. In this paper, we will describe a model system which we developed for testing the efficiency and the specificity of the phagemid subtractive hybridization technique. We also describe two simplified and efficient methods for preparing directional cDNA libraries which can be used in the subtractive hybridization procedure.

MATERIALS AND METHODS

Construction Of Phagemid Vectors

A series of phagemid vectors were constructed which were based on the pBluescript SK(−) and KS(−) vectors from Stratagene Inc, La Jolla CA. The standard cloning methods used to modify these vectors are described in references (9, 10), and (11). Four plasmids were constructed (figure 1). E61 is derived from pBluescript SK(−), and contains a polylinker domain whose sequence is shown in figure 2. Inserted between the Eco RI and Not I sites is a 0.95 kb cDNA fragment (DQβ2) encoding the b chain of the DQ allele from HLA-D. E145 is derived from pBluescript KS(−), and contains a polylinker domain shown in figure 2. E145 also has the DQβ2 cDNA inserted in the polylinker (but in an orientation opposite to that in E61). E10 is related to E61, but the DQβ2 fragment is replaced with the TN5 neomycin phosphotransferase gene (*neo*) (12). E11 is related to E145, but the DQβ2 fragment is replaced with the *neo* gene (in an orientation opposite to that in E10). Plasmid E4 is similar to E61,

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but does not have an insert in the polylinker domain (vector not shown).

Rescue and Purification Of Single-Stranded DNA

Single-stranded phagemid DNA was isolated from XL-1 bacteria (13) harboring the double-stranded form of the phagemid using the M13 rescue technique (14, 15, 16). Overnight bacterial cultures were diluted to an OD₆₀₀ of 0.05 (approximately a 1:100 dilution) into 450 ml of superbroth (35 g tryptone, 20 g yeast extract, 5 g NaCl/L, pH 7.5) and grown at 37°C to an OD₆₀₀ of 0.1–0.2 with good aeration. They were then infected at a multiplicity of infection of 10–20 with the R408 strain of M13 (17) for 6–9 hours (longer times of infection lead to more double-stranded DNA contamination in the single-stranded DNA preparation). Bacteria were removed by two rounds of centrifugation at 6000 rpm for 20 min. at 4°C in a Sorval GSA rotor, followed by vacuum filtration through Whatman #2 paper. The phage were precipitated by adjusting the solution to 4% polyethylene glycol (8,000 Daltons) and 0.5 M NaCl. The solution was incubated at 4°C for 2–4 hours, and centrifuged at 6000 rpm for 40 minutes at 4°C. The phage precipitate was dissolved in 0.5% SDS, 10 mM Tris HCl pH 7.4, 1 mM Na EDTA, 5 mM Na sulfite, 0.92 g KI/ml solution, and 80 µg ethidium bromide/ml solution. The phage lysate was centrifuged in a Beckman Ti50 rotor at 47,000 RPM, 20°C for 48 hours. Under these conditions, single-stranded DNA forms a fluorescent band below the double-stranded DNA and above the RNA. The single-stranded DNA fraction was removed using an 18 gauge needle and syringe and the sample was then centrifuged again using a Ti100 rotor at 70,000 rpm, for 18 hours, at 20°C. The single-stranded DNA was recovered, and was extracted with 1-butanol (9) to remove the ethidium bromide. The sample was diluted with two volumes of 10 mM Tris HCl pH 7.4 and 1 mM Na EDTA (TE), and the DNA was precipitated by adding six volumes of ethanol.

Residual double-stranded DNA was removed using two procedures: digestion with restriction endonucleases and magnesium-phenol extraction (18). To measure the efficiency of double-stranded DNA elimination, we added a marker plasmid (1 µg marker per 100 µg single-stranded DNA) which expresses either a neomycin resistance gene (E28, Rubenstein et al., manuscript in preparation) or a tetracycline resistance gene (pACYC184 (19)), but does not express the ampicillin resistance gene. In a typical reaction, 100 µg of single-stranded DNA was digested with the restriction endonucleases Bgl I and Pvu II (1–2 units of each enzyme per 1 µg single-stranded DNA) for 2–4 hours in 300 µL of 100 mM NaCl, 10 mM Tris HCl pH 7.4, 10 mM MgCl₂ and 1 mM DTT. Bgl I and Pvu II each cut the double-stranded vector at two sites; they did not cut single-stranded DNA as judged by agarose gel electrophoresis or by a DNA transformation assay. Siliconized microcentrifuge tubes (PGC Scientifics, Gaithersburg, MD) were used for all procedures involving single-stranded DNA. The reaction was stopped by adding SDS and EDTA pH 8.0 to final concentrations of 0.1% and 12.5 mM respectively. The proteins were removed by extracting with 150 µL of Tris HCl-buffered phenol pH 7.5 (9). The aqueous phase was saved and the phenol phase was extracted with 300 µL TE. Magnesium chloride was added to the pooled aqueous phases (600 µL) to a final concentration of 100 mM, and then 300 µL of Tris HCl-buffered phenol pH 7.5 was added, and the solution was vortexed. Under these conditions single-stranded DNA is insoluble, whereas double-stranded DNA

and RNA remain water-soluble. The sample was centrifuged at 16,000×g for 10 minutes to sediment the precipitated single-stranded DNA (it usually forms a pellet at the bottom of the tube, but occasionally remains at the phenol-water interface). The aqueous phase was removed and the phenol phase was then extracted (vortexing followed by centrifugation) two times with 300 µL of 100 mM MgCl₂ in TE to remove additional double-stranded DNA. The single-stranded DNA precipitate was then re-dissolved by adding 100 µL of 50 mM Na EDTA pH 8.0 to the phenol phase, followed by vortexing and centrifugation. The aqueous phase was collected, and the phenol phase and residual precipitate were then extracted two times with 150 µL of TE. Sodium acetate pH 6.5 (45 µL of a 3 molar solution) and ethanol (1 mL) were then added to the pooled aqueous phases (400 µL) to precipitate the DNA. The DNA was then subjected to another round of digestion with Bgl I and Pvu II followed by extraction with magnesium-phenol. Using this procedure we routinely reduced the amount of marker double-stranded DNA by a factor of 10⁶ and obtained >75% yield of the single-stranded DNA. The purified single-stranded DNA was stored in TE at 4°C.

Biotinylation Of Single-Stranded DNA

To biotinylate the single-stranded DNA we mixed 50 µL of 1 µg/µL solution of single-stranded DNA dissolved in water with 50 µL of 1 µg/µL long-arm Photoprobe® biotin (Vector laboratories, Burlingame, CA) in water (20). The solution was irradiated in an open tube placed in an ice bath 10 cm from a 275 watt sun lamp (General Electric) for 15 min. The single-stranded DNA was further biotinylated by adding 25 µL of long-arm photobiotin to the reaction and exposing the solution to an additional 15 minutes of irradiation. Biotins not covalently linked to the DNA were removed by adding 12.5 µL 1M Tris HCl pH 9.0, followed by 5 rounds of extraction with water-saturated 2-butanol. Sodium acetate pH 6.5 was added to a concentration of 0.3 M, and the biotinylated DNA was precipitated by adding two volumes of ethanol.

Hybridization Conditions

Each subtractive hybridization experiment consisted of a pair of samples. The control sample contained only target single-stranded DNA whereas the experimental sample contained both target and biotinylated driver single-stranded DNA. Also note that single-stranded R408 DNA (in about 10% of the target concentration) was present in all of the reactions.

Two different conditions were used for the hybridization reactions. In the first groups of reactions, 0.8 µg of biotinylated E145 (E145B) served as the driver and either E61 or E4 (0.1 µg), or E10 (0.05 µg) was the target in a 20 µL solution consisting of 0.2 M NaCl, 50 mM Na HEPES pH 7.6, and 2 mM Na EDTA. The sample was placed in a siliconized 650 µL tube and was covered with light-weight mineral oil (Sigma). It was heated to 95°C for two minutes, and incubated at 65°C for 11 hours.

In the second set of reactions, we used the following hybridization conditions: 1.8 µg of biotinylated E11 (E11B) was the driver and 0.2 µg of E10 was the target. Added to these reactions was 0.0025 µg of double-stranded pACYC184, which served as a control for DNA which would not be subtracted. These DNAs were dissolved in 11 µL of 0.5 M NaCl, 50 mM Na HEPES pH 7.6, 2 mM EDTA, and 40% deionized formamide. The samples were heated to 70°C for two minutes and incubated at 52°C for 21 hours.

Streptavidin-Phenol:Chloroform Extraction Of Biotinylated DNA

Removal of the biotinylated DNA, as well as DNA hybridized to the biotinylated DNA was achieved by the addition of streptavidin (Vector laboratory, Burlingame CA) followed by extraction with phenol:chloroform (1:1), using a modification of the protocol derived by Sive and St John (20). Following the hybridization, most of the mineral oil was removed, and the sample was diluted to 300 μ L by adding a 37°C solution of 500 mM NaCl, 50 mM HEPES pH 7.6, 2 mM Na EDTA, and 15 μ g/mL of streptavidin for 2 minutes. Streptavidin-biotin-DNA complexes were extracted by adding 200 μ L of a 37°C solution of phenol:chloroform (1:1) pH 7.5, vortexing for 5 seconds, and centrifuging for 5 minutes at room temperature at 16,000 $\times$ g. Samples were extracted at least three times with phenol:chloroform, and the organic phases were re-extracted with TE to optimize recovery of the DNA. The DNA in the aqueous phase was ethanol precipitated, and redissolved in TE.

Agarose Gel Electrophoresis Of Single-Stranded DNA

Following the subtractive hybridization, samples were analyzed by electrophoresis in horizontal 0.8–1.0% (w/v) agarose gels ('SeaKem', FMC, Rockland ME) using either TBE or TAE buffer (9) containing 0.3 μ g/mL ethidium bromide at 3 volts/cm.

Enzymatic Conversion Of Single-Stranded DNA To Double-Stranded Form

Thermus aquaticus (Taq) DNA polymerase and a single-stranded primer homologous to the polylinker domain were used to convert the single-stranded DNA to double-stranded form. In a typical reaction, 100 ng (0.08 pM) or less of target DNA was dissolved in 30 μ L of Taq buffer [50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM MgCl₂, and 0.01% (w/v) gelatin (Kodak)], containing 30 ng (4 pM) of the primer oligonucleotide (5'-GAGCTCCTC-GAGGTCTAGAGGCT-3'). The solution was heated to 95°C for 1 minute and then incubated at 55°C for 30 minutes to anneal the oligonucleotide to the single-strand phagemid DNA. 30 μ L containing 0.2 mM of dATP, dTTP, dCTP and dGTP, 0.5 units Taq DNA polymerase (Cetus) in Taq buffer (solution preheated to 55°C), was added to the sample for 2 minutes, and then heated to 70°C for 10 minutes. The reaction was stopped by adding 0.1% SDS and 12.5 mM Na EDTA pH 8.0 to the solution, followed by extraction with 40 μ L of phenol-chloroform (1:1). The organic phase was extracted with 35 μ L of TE, and the aqueous phases were pooled (100 μ L). The DNA was precipitated by adding either 5 μ g of linear polyacrylamide or 10 μ g of glycogen (Boehringer Mannheim), 100 μ L of 4M NH₄Ac pH 7.0, and 400 μ L of ethanol. The pellets were washed two times in 80% ethanol and dried before being dissolved in aqueous solvent.

In subtractive hybridization experiments we added a marker single-stranded DNA to both the experimental and control samples before the Taq polymerase reactions. The marker DNA, which could be distinguished from the target DNA on the basis of antibiotic resistance, served as a control for the efficiency of single to double-strand conversion. We used a bacterial transformation assay to determine the efficiency of double-stranded conversion in the samples.

Transformation Of Bacteria By Electroporation

We used the protocol described by Dower et al (21). Typically, 3 μ L of DNA in 2 mM Tris HCl pH 7.4 and 0.2 mM Na EDTA

were added to 45 μ L of 'electrocompetent' MC1061 strain of *E. coli* (prepared as in (21)). A Biorad Gene Pulser apparatus was used, and was set at 25 uF, 2.5 kV and 400 ohms. Following discharge of the electric pulse, 1 ml SOC media (21) was immediately added to the bacteria, and they were then grown for 1 hour at 37°C. The bacteria were then plated on LB agar containing either ampicillin (50 μ g/ml), tetracycline (12 μ g/ml) or kanamycin (30 μ g/ml).

Isolation of Poly A⁺ RNA

Mouse forebrain and whole rat brain were rapidly dissected after sacrifice, frozen on dry ice, and stored at -70°C prior to preparation of total RNA by the guanidine-thiocyanate/cesium chloride ultracentrifugation method (9). Poly A⁺ RNA was prepared by chromatography on oligo-dT cellulose (Type III, Collaborative Research, Bedford MA) (9).

Adaptor-Primer cDNA Synthesis Method

Vector Preparation

Not I-Blunt vector was prepared by digesting E61 with Not I (Stratagene) and EcoR V (New England Biolabs, Beverly MA) followed by treatment with calf alkaline phosphatase (Molecular Biology Grade, Boehringer Mannheim, Indianapolis IN). The 2.9 kb vector fragment was purified by electrophoresis in Sea Plaque low-gelling temperature agarose (FMC Bioproducts, Rockland ME).

cDNA Synthesis By The Adaptor-Primer Method

Six micrograms of poly A⁺ RNA was mixed with 400 pmoles of a Not I adaptor-primer, heated to 65°C for 2 minutes, and then cooled on ice for 2 minutes. The 28 base adaptor-primer, which had the following sequence: 5'-d(AATTCGCGGCCGCTTTTTTTTTTTTTT)-3', was obtained from Promega (Madison WI). First and second-strand cDNA synthesis was performed using standard methods (22, 23, 24) using AMV reverse transcriptase (Molecular Genetic Resources, Tampa FL) RNaseH (Promega), *E. coli* DNA polymerase (New England Biolabs), and *E. coli* DNA ligase (New England Biolabs). At the end of the reaction residual RNA was degraded by adding 10 μ g RNase A (Boehringer Mannheim) for 30 minutes at 25°C. The DNA was precipitated using 3mM spermine following the procedure of Hoopes and McClure (25). The DNA was then treated with *E. coli* DNA polymerase, Klenow fragment (New England Biolabs), phenol extracted and ethanol precipitated. Next the DNA was treated with Not I and then loaded on a Sepharose CL-4B (Pharmacia, Piscataway NJ) column (17 $\times$ 0.5 cm) which was equilibrated with 100mM NaCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA. Fragments ~0.4 kb and greater were pooled, ethanol precipitated and resuspended in 40 μ L of 2 mM Tris-HCl pH 7.4 and 0.2 mM Na EDTA. The concentration of the cDNA was 30 ng/ μ L. Approximately 120 ng (15%) of the cDNA was ligated into Not I-EcoR V digested E61 vector (180ng) in 30 μ L containing 800 units T4 DNA ligase (New England Biolabs), 1mM ATP and ligase buffer (50mM Tris-HCl, pH 7.8, 10mM MgCl₂, 20mM DTT) for 20 hours at 15°C.

Vector-Primer cDNA Synthesis Method

Vector-Primer Preparation

To prepare the vector-primer E61 was digested with Not I, and treated with calf alkaline phosphatase. The reaction was stopped with SDS/EDTA, phenol extracted twice and ethanol precipitated. A Not I-(T)₁₅ 21mer oligonucleotide with sequence:

5'-d(GGCCGCTTTTTTTTTTTTTT)-3' was synthesized on a Biosearch model 8600 DNA synthesizer, purified by acrylamide gel electrophoresis, and phosphorylated using T4 polynucleotide kinase. The Not I-(T)₁₅ oligonucleotide was ligated to the Not I digested vector in 0.3 ml total volume containing 24 µg of Not I-(T)₁₅ oligo, 1mM ATP, 12 Weiss units of T4 DNA ligase (Stratagene) and ligase buffer for 20 hours at 15°C. This reaction was stopped with SDS/EDTA, phenol extracted, ethanol precipitated and then digested with EcoR I (BRL, Bethesda MD) and calf alkaline phosphatase. The vector was purified by electrophoresis in 1% low-gelling temperature agarose. Vector-d(T)₁₅ was further purified by chromatography on oligo-dA cellulose.

cDNA Synthesis By Vector-Primer Method

One microgram of rat brain poly A⁺ RNA was heated to 70°C for 5 minutes, cooled on ice, and added to a 50µl reaction mix containing 250 ng vector-primer. First and second-strand cDNA synthesis were performed as in the previous section except the RNase A step was omitted. After spermine precipitation, the DNA was treated with the Klenow fragment of *E. coli* DNA polymerase. The cDNA was extracted with phenol and ethanol precipitated after addition of NH₄-acetate and 20µg glycogen. The vector-cDNA was ligated in a final volume of 0.5ml containing 16 Weiss units T4 DNA ligase (Stratagene).

RESULTS

Construction Of Phagemid Vectors

We constructed a series of phagemid vectors that are based on the pBluescript SK(-) and KS(-) vectors from Stratagene Inc. pBluescript has the *colE1* (from plasmid pUC) origin of replication, the ampicillin resistance gene, a β -galactosidase promoter and truncated coding sequence, T3 and T7 promoters, a polylinker domain and the *f1* origin of replication. We modified the polylinker to minimize complementarity between the single-stranded forms of the SK(-) and KS(-) vectors in order to prevent hybridization between the vectors during the subtractive hybridization. We also inserted two different DNA fragments (stuffers) in opposite orientations into the polylinker domain. The structure of these vectors are shown in figure 1. One pair of vectors, E61 and E145, contain a 0.9 kb stuffer fragment encoding the β chain of the DQ allele from HLA-D (26). E61 and E145 were also designed to permit directional insertion of cDNAs (or any other DNA fragment) with a Not I and a blunt end in the place of the DQ β stuffer fragment. Figure 1 also shows another pair of vectors, E10 and E11, which contain a 1.3 kb stuffer fragment encoding the neomycin phosphotransferase gene (*neo*) (12). Figure 2 shows the sequence of the polylinker domains in E61 and E145.

Production of Single-Stranded Phagemids

We used the M13 rescue method (14, 15, 16) to produce the phagemids in single-stranded form. We introduced several useful steps in the isolation of the single-stranded DNA which proved necessary to obtain a preparation essentially free of contaminant double-stranded DNA. The nature of the subtractive hybridization procedure described in this paper requires that there be virtually no double-stranded phagemid DNA contaminating the single-stranded DNA sample. The first novel step in the purification protocol was a KI density gradient which separated single-stranded DNA from RNA and double-stranded DNA. Two

rounds of centrifugation typically result in single-stranded DNA which has less than 1% double-stranded phagemid contamination. Residual double-stranded DNA is eliminated with two cycles of digestion with Pvu II and Bgl I, followed by selective precipitation of the single-stranded DNA using a magnesium-phenol protocol (see materials and methods). Following this procedure we could verify that there was less than 1 double-stranded phagemid

Figure 1: Diagram describing the structures of the phagemid vectors. Abbreviations: AMP^R: ampicillin resistance gene, arrow depicts direction of transcription; pUC ori: *colE1* origin of replication from the pUC vector; f1(-): *f1* origin of replication, arrow depicts direction of *f1* replication; Kpn I, Sal I, EcoR I, Sac I, Not I, Hind III, Sfi I, Bgl II and EcoR V mark sites that are cut by the corresponding restriction endonuclease; kb: kilobase. The thin line corresponds to the vector sequences; the white boxed-in area corresponds to the polylinker domain; the black boxed-in area corresponds to the stuffer fragments. Plasmids E61 (A) and E145 (B) have the DQ β 2 stuffer fragment inserted in opposite orientations in their polylinker domains. E10 (C) and E11 (D) have the *neo* gene inserted in opposite orientations in their polylinkers. Kpn I/Sal I and Kpn I/EcoR V signify that there has been a deletion between these sites; note that only the Sal I site remains intact.

Figure 2: Nucleotide sequence of the polylinkers in E61 and E145. STUFFER represents the position of the 0.9 kb DQ β cDNA which is inserted between the Not I and EcoR I sites. Sac I, Xho I, Xba I, Sfi I, Sac II, Not I, EcoR I, EcoR V, Hind III, Cla I, Sal I and Bst XI signify the nucleotide sequence recognized by the corresponding restriction enzyme, and the arrow points to the site of phosphodiester cleavage. 5' and 3' define the polarity for the given sequence. The sequence was determined based upon the known sequence of polylinker from pBluescript, and from restriction enzyme digests of E61 and E145.

molecule per 10^7 single-stranded molecules (see material and methods).

Agarose Gel Electrophoresis Of The Single-Stranded DNA

Agarose gel electrophoresis separates the various components of the single-stranded DNA preparation. Figure 4A shows an ethidium bromide stained agarose gel of a single-stranded phagemid preparation of E61 DNA. Two DNA species are resolved on the gel. The slower migrating is the 6.4 kb single-stranded M13 DNA (R408 strain). The faster migrating molecule is the single-stranded 3.8 kb E61 DNA. Biotinylation of the single-stranded DNA does not significantly alter its mobility (results not shown).

Bacterial Transformation With Single-Stranded DNA

We measured the efficiency with which the purified single-stranded DNA transforms bacteria by electroporation. The transformation efficiency of single-stranded DNA depends on the amount of DNA used in the transformation (see Table 1). For instance, transformation of MC1061 bacteria with 1 ng produced

9×10^5 transformants/ μg whereas transformation with 100 ng produced 6×10^6 transformants/ μg . In contrast, the transformation efficiency of double-stranded DNA remains constant over this range of DNA concentrations (Table 1 and Dower et al (21)). Also note that double-stranded DNA transformation efficiency is ~ 100 to 1000 fold greater than single-stranded DNA, depending upon the concentration of DNA. Biotinylation of single-stranded DNA decreases its transformation efficiency by a factor of $> 10^5$. Conversion of single-stranded

Figure 3: Flow-chart describing the subtractive hybridization scheme. Four circular DNA structures are represented; E11, E10 and R408 are single-stranded DNA molecules; pACYC184 is a double-stranded DNA plasmid. The size of each molecule is listed in kilobases (kb). NEO^R and TET^R represent genes encoding enzymes which confer bacterial resistance to neomycin and tetracycline, respectively. Biotinylation of E11 is represented by the letter B attached to the E11 circles. During the hybridization reaction, E11 and E10 form a partial duplex molecule due to base pairing in the regions of their stuffer fragments. Streptavidin is added to the reaction, which binds to the biotinylated molecules; the DNA-protein complexes are removed from solution using a phenol:chloroform extraction procedure. The molecules which remain in the aqueous phase are then either directly examined using agarose gel electrophoresis, or are converted to double-stranded form, and transformed into bacteria.

Figure 4: Qualitative determination of the efficiency and specificity of subtractive hybridization. The photographs are of DNA-ethidium bromide fluorescence from agarose gels. A, B and C correspond to three different experiments which are described in the text. A: subtractive hybridizations with (+) and without (-) 0.8 μg biotinylated single-stranded E145 driver and with and without 0.1 μg of single-stranded E61 and $\sim 0.01 \mu\text{g}$ R408 target. B: subtractive hybridizations with and without 1.8 μg biotinylated E11 driver and 0.2 μg E10 and $\sim 0.02 \mu\text{g}$ R408 targets. One half of each reaction was electrophoresed. C: subtractive hybridization with and without 0.8 μg E145B and with either 0.1 μg E4 or 0.05 μg E10 target. The sizes of the various single-stranded DNAs (in kilobases, kb) are listed along the sides of the photographs.

Table 1: The Efficiency* of DNA Transformation Using Single-Stranded or Double-Stranded DNA.

Nanograms Electroported	100	50	20	10	5	1	0.1
Single-Strand Efficiency * ($\times 10^{-6}$)	6	3	1	0.9	0.6	0.9	ND
Double-Strand Efficiency * ($\times 10^{-8}$)	8	ND	ND	9	ND	10	10

*Efficiency is defined as the number of ampicillin resistant bacteria that are produced per microgram of DNA that is electroporated into MC1061 bacteria. DNAs were electroporated as described in the materials and methods. ND indicates that the sample was not done.

DNA to double-stranded form using Taq DNA polymerase increases the transformation efficiency several hundred fold. Ligation of the nicks in the DNA after the Taq polymerase reaction did not increase the transformation efficiency. We also found that 5 μ g of glycogen does not inhibit electroporation of either single-stranded or double-stranded DNA, whereas 5 μ g of linear polyacrylamide inhibits the efficiency of double-stranded DNA by 6-fold and single-stranded DNA by 25-fold.

Subtractive Hybridization Using A Three Phagemid System

To determine the efficiency of subtractive hybridization using single-stranded phagemids with directional inserts we performed subtractive hybridizations using biotinylated single-stranded E145 as the driver and E61 as the target. Single-stranded R408 DNA (in roughly 10% of the amount of the target DNA) was also present in these reactions. R408 DNA should not be complementary to either the driver or the target, and thereby serves as a control for the specificity of the subtraction. The DNA sequence of E145 and E61 is complementary in the stuffer fragment domain; therefore, during the hybridization reaction E145 and E61 should hybridize to each other only in the region of their stuffer fragments. Following the hybridization incubation, the biotinylated E145 DNA, and any E61 DNA that has hybridized to it, is removed from solution by adding streptavidin and then extracting with phenol:chloroform using the protocol of Sive and St. John (20).

Figure 4A shows an agarose gel on which the products of the subtractive hybridization were electrophoresed. The first lane shows that extraction of the hybridization reaction containing 0.8 μ g of biotinylated E145 and 0.1 μ g of E61 resulted in removal of the biotinylated E145 and the E61, but not of the single-stranded R408 DNA (thereby demonstrating the specificity of the subtraction). The second lane shows that extraction of a sample of E61 (without biotinylated E145) does not remove E61. Finally, the third lane shows that extraction of a sample containing only biotinylated E145 removes virtually all of the DNA (no fluorescence is seen). Typically, the extraction procedure results in roughly a 50% non-specific loss of DNA (data not shown).

To further verify the specificity of the subtractive hybridization, we prepared solutions containing 0.8 μ g of biotinylated E145 driver, and a single-stranded phagemid target consisting of either 0.1 μ g E4 (vector without stuffer insert), or 0.05 μ g E10 (vector with *neo* gene insert). Figure 4C shows the results of these subtractive hybridizations. In contrast to the experiments with the E61 target, neither the E4 nor the E10 DNA was removed by the subtraction procedure.

Bacterial Transformation Assay Of Subtractive Hybridization Efficiency

A more sensitive and quantitative measure of the efficiency of the subtractive hybridization is to determine the amount of target

Table 2: Quantitation of Subtractive Hybridization Using a DNA Transformation Assay*

Sample	KAN	TET	TET/KAN	TET ENRICHMENT
Subtracted	1×10^2	5×10^3	5×10^1	5×10^3
Control	3×10^5	3×10^3	1×10^{-2}	

*The assay involves electroporation of DNA following the subtractive hybridization. The single-stranded phagemids have been converted to double-stranded form. KAN and TET refer to the total number of kanamycin and tetracycline resistant bacteria that were produced by the electroporation. TET/KAN is the ratio of tetracycline to kanamycin colonies. TET enrichment is calculated by dividing the subtracted TET/KAN ratio by the control TET/KAN ratio; it represents the relative amount that E10 (KanR) was reduced relative to pACYC184 (TetR), and is therefore a measure of the subtraction. The subtracted sample contained both driver (E11B) and target (E10), and the control sample contained only E10. See text for details.

remaining after the subtraction using a bacterial transformation assay. Figure 3 is a diagram which describes the steps in this experiment. We performed a subtraction between 1.8 μ g of biotinylated E11 driver and 200 ng of E10 target in 11 microliters. We added 2.5 ng of pACYC184 double-stranded plasmid to the hybridization sample as an internal standard (pACYC184 will not hybridize to the E11 driver). pACYC184 carries genes for tetracycline and chloramphenicol resistance, and therefore can be distinguished from E10, which carries genes for both ampicillin and kanamycin resistance. The hybridization was performed using 40% formamide, which permitted decreasing the temperature to 52°C. The lower temperature significantly decreases the frequency of strand breakage (data not shown).

Following the hybridization and extraction procedures, half of the sample was analyzed by agarose gel electrophoresis. Figure 4B shows that the E10 target was subtracted, whereas the R408 DNA was not.

Ten percent of the DNA was then converted to double-stranded form using the Taq DNA polymerase reaction. Taq DNA polymerase was chosen because the reaction could be performed at elevated temperatures which would reduce the amount of non-specific priming. The DNA polymerase reaction products were electroporated into MC1061 bacteria. The bacteria were plated on kanamycin and tetracycline plates. The tetracycline plates measured the number of pACYC184 plasmids, serving as a control for non-specific losses during the procedure and for transformation efficiency. The number of kanamycin colonies corresponded to the number of E10 phagemids. We normalized the samples with respect to the number of tetracycline transformants, and then compared the number of kanamycin colonies in the control and subtracted samples (see Table 2). We found that one round of subtractive hybridization, resulted in a 5000-fold specific reduction in the amount of E10 DNA. We

Figure 5: Scheme for preparation of cDNA libraries by the Adaptor-Primer procedure. Experimental details are presented under Materials and Methods and under Results.

analyzed the structure of the phagemids at the end of the procedure using agarose gel electrophoresis of restriction digests of the DNA. We found no evidence for changes in the size of the plasmids (data not shown).

cDNA Library Construction Using The Adaptor-Primer Method

We have developed two simplified methods for constructing directional cDNA libraries; an adaptor-primer and a vector-primer procedure. For both of these protocols we used the E61 vector. To prepare the vector for the adaptor-primer procedure, E61 was digested with Not I and EcoR V (which leaves a blunt-end), and the 5' terminal phosphates were removed using calf alkaline phosphatase (to decrease the amount of vector self-ligation).

Figure 5 illustrates construction of a directional cDNA library using an adaptor-primer and ligation into a vector with Not I cohesive and blunt ends. Poly A⁺ RNA was annealed to a Not I adaptor-primer and first-strand cDNA synthesis performed using reverse transcriptase. Second-strand synthesis was accomplished using E. coli DNA polymerase, E. coli DNA ligase and RNase

Table 3: Efficiency of cDNA library construction using the Adaptor-Primer and Vector-Primer procedures.

Procedure	Independent Recombinants Per Microgram A ⁺ RNA	
	MC1061	XL-1
Adaptor-Primer	8×10^7	4×10^6
Vector-Primer	ND	3×10^7

Six micrograms of mouse telencephalon poly A⁺ RNA was used for cDNA synthesis and library construction by the adaptor-primer procedure and 7.5% of the cDNA was used for each transformation reaction. One microgram of rat brain poly A⁺ RNA was used for library construction by the vector-primer procedure and 25% of the material used in the transformation reaction. ND, not determined (20 fold greater yield is expected with MC1061 bacteria, however XL-1 bacteria were used so single-stranded rescue could be performed).

H (22, 23). To ensure that staggered ends of the double stranded cDNA were converted to blunt ends a separate reaction was performed using the Klenow fragment of E. coli DNA polymerase. The DNA was then digested with Not I and size-selected on a Sepharose-CL4B column. The cDNA, which at this stage has one Not I cohesive end and one blunt end, was ligated into the vector which also has a Not I and a blunt end. Fifteen percent of the cDNA generated in this experiment ($\sim 0.12 \mu\text{g}$) was ligated to $0.18 \mu\text{g}$ of vector. There were 4×10^7 primary recombinants using MC1061 bacteria and 2×10^6 primary recombinants using XL-1 bacteria. Therefore, efficiencies of 8×10^7 and 4×10^6 recombinants per microgram poly A⁺ RNA were obtained using MC1061 and XL-1 bacteria respectively (Table 3).

cDNA Library Construction Using The Vector-Primer Method

Construction of the vector-primer is diagrammed in figure 6. E61 was linearized by cutting at the Not I site within the polylinker domain and then ligated to a synthetic oligonucleotide consisting of fifteen 3' thymidine residues and a 5' sequence complementary to and capable of regenerating the Not I site. The stuffer fragment was removed from the vector by digestion with EcoR I and agarose gel electrophoresis, producing a 2.9kb vector with a 15 thymidine residue overhang at one end. The vector-primer was further purified by chromatography on oligo-dA cellulose to remove vector without ligated oligonucleotide. The vector was treated with alkaline phosphatase prior to ligation of the adaptor oligonucleotide to prevent vector-vector ligations, and also after digestion with EcoR I to reduce circularization of the vector with no attached cDNA.

The vector-primer library construction scheme is shown in figure 7. One microgram of rat brain poly A⁺ RNA was annealed to 250ng of the vector-primer and first-strand synthesis was performed using reverse transcriptase. Second-strand replacement was performed using E. coli DNA polymerase, E. coli DNA ligase and RNase H. To ensure that both the EcoR I and cDNA ends were blunt a separate reaction using the Klenow fragment of E. coli DNA polymerase was performed. The linear vector-cDNA molecules were then circularized using T4 DNA ligase. Following the circularization step bacteria were transformed with the DNA by electroporation. One-fourth of the reaction described above was used to transform XL-1 bacteria. There were 7×10^6 independent recombinants, corresponding to 3×10^7 recombinants per microgram of starting polyA⁺ RNA (Table 3). This is eight-fold more efficient than the adaptor-primer method.

Figure 6: Scheme for preparation of the Vector-Primer. Experimental details are presented under Materials and Methods and under Results. The names of restriction endonucleases underneath vector diagrams indicate cohesive ends generated by digestion with that enzyme. Note I-(T)₁₅ corresponds to the oligonucleotide: 5'-d(GGCCGCTTTT TTTTTTTTTT)I₁₅-3'.

cDNA Library Analysis

Purified plasmid DNA from the adaptor-primer and vector-primer libraries was linearized by digestion with Sal I (there is one Sal I site, which is within the polylinker of the phagemid vector 5' to the cDNA inserts) and analyzed by agarose gel electrophoresis. There is a smooth distribution of DNA extending upward from the size of the vector (2.9kb) to about 8kb indicating that both libraries contain cDNA inserts ranging up to at least 6kb (data not shown). In the vector primer library, 3% of the plasmids either have no insert or are smaller than vector-size (2.9 kb). DNA smaller than 2.9 kb appears to result from exonucleolytic digestion of the vector since the restriction sites nearest the ends of the vector are lost in those molecules. In the adaptor-primer library there is virtually no vector size DNA suggesting that plasmids without inserts comprise <1% of the library.

The orientation of the inserts in the vector-primer library are defined because the oligo-dT primer is part of the vector. To verify that the inserts in the adaptor-primer library were directionally oriented the DNA was digested with Not I. More than 95% of the plasmids were cut (data not shown) demonstrating that the Not I site was regenerated following

Figure 7: Scheme for construction of cDNA libraries by the Vector-Primer procedure. Experimental details are presented under Materials and Methods and under Results. The starting vector is prepared as shown in Figure 6.

ligation of the cDNA to the vector. Therefore, because the Not I site is adjacent to the 3' end of the cDNA at least 95% of the inserts are directionally oriented.

Based on the size range of the libraries we thought it likely that both contained inserts corresponding to the full coding length of mRNA's. cDNA clones have been isolated from the mouse forebrain library which encode the entire coding sequence for the mouse serotonin 5HT_{1a} (2.2kb), adrenergic β_1 (3kb) and adrenergic α_1 (1.8kb) receptors (27). We have also shown using southern analysis that the vector-primed (rat brain) library has full-length preproenkephalin cDNA clones (data not shown).

DISCUSSION

In this paper we have described a protocol to perform subtractive hybridization with phagemid vectors containing directional inserts serving as both the driver and the target. First, we described the construction of a set of phagemid cloning vectors, based on pBluescript, which are designed to allow the directional insertion of DNA sequences. We eliminated significant complementarity in the polylinker sequences to prevent hybridization of the vectors simply on the basis of complementarity in their polylinker domains. Next, we developed a rigorous protocol for purifying single-stranded DNA using the novel steps of KI density gradients, restriction enzyme digestion, and magnesium-phenol

extraction. These steps are essential because trace contaminants of double-stranded phagemid destroy the efficacy of the subtractive hybridization. We performed hybridizations using a model system containing four species of nucleic acid: (1) 'driver': biotinylated single-stranded phagemid DNA; (2) 'target': single-stranded phagemid DNA containing a stuffer fragment in an anti-parallel orientation to the stuffer in the driver DNA; (3) 'control' single-stranded DNA, which is not homologous to the driver; (4) 'control' double-stranded DNA, which has a drug-resistance marker different than the phagemid. With this model system we determined the efficiency of the subtractive hybridization using both a transformation and an electrophoresis assay. The transformation assay is much more sensitive, and it showed that one round of hybridization resulted in a 5000-fold subtraction. We also demonstrated the specificity of the subtraction by showing that single-stranded phagemid DNA, which does not contain a stuffer fragment homologous to the driver's stuffer fragment, is not extracted.

Certain important aspects of our protocol have been previously demonstrated by other researchers. Duguid and co-workers (5, 8) were the first to use phagemid vectors for subtractive hybridization and to show that target molecules, which is already in a cloning vector, have significant advantages over the traditional method of having to ligate nanogram amounts target sequences into a vector following the subtractive hybridization. Sutcliffe and co-workers (7) showed that having driver sequences in cloned form, circumvents the problem of not having a large and easily prepared supply of mRNA. Finally, we adopted the streptavidin-phenol:chloroform method of Sive and St. John (20) for the removal of the biotinylated driver and the driver:target hybrids.

Although our method has similarities to other subtraction procedures, it also has several important differences. First, our method uses inserts which are directionally oriented. This has important ramifications in subtractive hybridizations between cDNA libraries. If the inserts are unidirectional in each library, then the only hybridization reactions which can occur are between complementary molecules in the different libraries. This is not the case if the cDNAs are bidirectionally inserted in the cDNA libraries; in this case the efficiency of subtraction will decrease because some of the target molecules will hybridize to complementary target molecules; hence they will become unavailable for hybridization to the driver, and therefore will not be subtracted.

Furthermore, directional inserts should eliminate the need to use a large ratio of driver to target in the reaction. Hence, the rate should increase by a factor of ~ 10 if one increases the ratio of the target to driver from 1:20 to 1:2 (while holding the driver concentration constant). A ten-fold increase in reaction rate could be significant when performing subtraction between complex cDNA libraries, where a CoT of > 1000 may be needed to obtain efficient subtraction of rare cDNAs. We are presently testing this hypothesis.

Finally, directional inserts also permit increased specificity in converting the single-stranded DNA into double-stranded form. For instance, we use an oligonucleotide primer (for the DNA polymerase reaction) which bridges the polylinker-poly A tract. This primer will only be fully complementary to single-stranded phagemids which have the proper polylinker, and which have a cDNA insert in the appropriate orientation. Hence, single-stranded phagemids which either have the wrong polylinker (such as the driver phagemids) or which have a cDNA insert in the

wrong orientation, or which do not have a cDNA insert, will not be efficiently converted to double-stranded form.

As noted earlier, we have gone to great lengths to remove double-stranded phagemid DNA from our single-stranded preparation. Using single-stranded DNA preparations described by other workers, we found that although the material appeared pure by agarose gel electrophoresis, it did contain double-stranded DNA when tested by digestion with double-strand specific restriction endonucleases and the bacterial transformation assay. Double-stranded DNA transforms bacteria between 100 and 1,000-fold more efficiently than single-stranded DNA (depending upon the amount of single-stranded DNA; see table 1). Therefore small amounts of double-stranded DNA in a target preparation (as little as 0.01%), confounds the subtractive hybridization procedure.

We have also developed a simple and sensitive method to determine the efficiency of the subtraction using a bacterial transformation assay. During the hybridization, we add a small amount (1% of the target concentration) of a plasmid which expresses a drug resistance marker to either kanamycin (E10 and E28) or to tetracycline (pACYC184). In experiments not described in this paper, we have used both double-stranded (E28) and single-stranded (E10) forms of the kanamycin marker plasmids. After the subtractive hybridization is completed, we measure the efficiency of subtraction by determining the ratio of ampicillin resistant colonies (which corresponds to the target) to either kanamycin or tetracycline containing colonies.

We use hybridization conditions which improved the recovery of the target DNA. Although single-stranded DNA is less sensitive to strand scission than is single-stranded RNA, it is less stable than double-stranded DNA. We found that by adding formamide to the reaction, we were able to perform the hybridizations at 52°C. At this temperature, we observed significantly less degradation of the single-stranded DNA. Biotinylation of the driver, through an unknown mechanism, promotes strand breakage during the hybridization incubation. This is fortuitous for several reasons. First, strand breakage produces smaller driver fragments, which hybridize at a faster rate than larger fragments (11). Secondly, hybridization between two single-stranded circles is likely to be inefficient for topological reasons.

We are in the process of using these methods to perform subtractive hybridization between directional cDNA libraries made from mouse telencephalons at different developmental stages. To this end, we have developed two simplified and efficient methods to produce directional cDNA libraries. There are two general approaches for directional cDNA cloning. In one, poly A⁺ RNA is primed from an adaptor-primer which generates a restriction site at one end of the cDNA. After second-strand synthesis adaptor oligonucleotides with a cohesive end different from the restriction site within the adaptor-primer are ligated onto both ends of the cDNA (24, 28, 29, 30). We have simplified this method by showing that adaptor ligation is unnecessary, and that cloning of cDNA's with one cohesive end and one blunt end is both efficient and directional.

The second established method for directional cDNA synthesis is to use the vector as the primer (23, 31). However, we find that it has several steps which can be eliminated, thus significantly simplifying the procedure. We have replaced the terminal transferase tailing reaction with ligation of a synthetic oligonucleotide of defined length. We have also eliminated the step in which the joining fragment is ligated to the vector-cDNA

fragment. In doing so a bimolecular ligation is converted to a cyclization reaction (which is more efficient). Another significant advantage of the vector-primer method is that the cDNA is never exposed to a restriction enzyme. Thus there is no potential for loss of clones that contain the restriction site used to create the asymmetric ends, as may occur with any of the other unidirectional cloning procedures. While this manuscript was in preparation, a similar vector-based cDNA cloning method was described (32).

Using the directional cDNA cloning and subtractive hybridization methods described in this paper, we have performed a subtractive hybridization between mouse adult and embryonic forebrain cDNA libraries. We have isolated seven cDNAs which correspond to genes which are preferentially expressed in the mouse embryonic forebrain (Porteus, Brice, Usdin, Ciaranello and Rubenstein, manuscript in preparation).

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Mental Health (MH39437), and the John Merck Fund. J.L.R.R. is a recipient of grants from the Scottish Rite Foundation and the Andre and Bella Meyer Fund. A.E.J.B. is in the Stanford Medical Scholars program. R.D.C. is the recipient of a Research Scientist Award from the National Institute of Mental Health (MH00219). M.H.P. is the recipient of a M. D. Ph. D. fellowship from Merck, Sharp and Dohme Research Laboratories and is a student in the Medical Scientist Training Program at Stanford University. T.B.U. is the recipient of a fellowship from the Dana Foundation. We thank Jack Barchas for providing support to T.B.U. through a National Institute of Drug Abuse Grant (DA05010). We are also grateful to Irving Weissman, Andrew Zelentz, John Elliott, Jeff Vieira and Douglas Brutlag for their help and advice during the development of this work.

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