Kindred DNA Amplification from Two Distinct Populations of cDNA Fragments

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ABSTRACT

Kindred DNA amplification is a novel and cost-effective method developed to isolate common cDNA fragments between two distinct cDNA populations. Unlike subtractive hybridization, which discards common sequences, kindred DNA amplification isolates and amplifies these sequences within a single hybridization procedure. The utility of this method is demonstrated by cloning the genes in common between two different but metabolically homologous muscles, murine ventricular myocardium and soleus. The reliability of kindred DNA amplification was confirmed by Southern hybridization.

INTRODUCTION

A vast number of biological processes, such as pre- and postnatal development differentiation, energy production, disease adaptation, and protection underscore the function of mammalian systems. Each of these processes is governed by specific gene expression that is regulated by two principal mechanisms. Differential expression operates via variations of sets of expressed genes. The second mechanism involves alterations in the expression level of an individual gene (epigenetic changes). At present, several methods based on cDNA subtraction are available for characterization of differential gene expression (1-3), whereas quantitative PCR together with micro- and macroarray techniques enables assessment of the epigenetic changes (4-6). In some cases, however, it is necessary to define the sequences in common between differentially expressed genes, for instance, in identifying genes specific to colon cancer (4). Then, time-intensive characterization of individual DNA populations followed by computer analysis to define the sequence differ-

Table 1. Oligonucleotides and Adapters Used in the Study

Primer Name	Primer Sequence (5'→3')
SMART	AAGCAGTGGTAACAACGCAGAGTACG CGGG
SMART CDS primer	AAGCAGTGGTAACAACGCAGAGTACTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTT (A,C,G)(A,C,G,T)
5' PCR primer	AAGCAGTGGTAACAACGCAGAGT
Adapter 1 long oligonucleotide	CTAATACGACTCACTATAGGGCTCGAG CGGCCGCCCGGGCAGGT
Adapter 1 short oligonucleotide	ACCTGCCCGG
Adapter 2R long oligonucleotide	CTAATACGACTCACTATAGGGCAGCG TGGTCGCGGCCGAGGT
Adapter 2R short oligonucleotide	ACCGCGGCCG
PCR primer 1	CTAATACGACTCACTATAGGGC
Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested PCR primer 2R	AGCGTGGTCGCGGCCGAGGT

ences has been used (4-6). We report on kindred DNA amplification, which is a novel and simple method to identify and select common DNA fragments between pools. Kindred DNA amplification utilizes DNA hybridization with subsequent suppression PCR (7,8), using modifications enabling one to selectively suppress or enhance the genes of interest. We have applied this technology to isolate the genes in common between two different muscles (myocardium and soleus), which are known to share mutual properties—predominantly dependence on the aerobic energy metabolism and regulation of mitochondrial function distinct from that of glycolytic muscles (9). This application stemmed from studies that demonstrated the low apparent affinity of mitochondrial respiration to exogenously added ADP in oxidative versus glycolytic muscles (extensor digitorum longus) (10,11). Selective proteolysis resulted in increased affinity of mitochondria to ADP in oxidative muscles but exerted no effect on that parameter in glycolytic muscles (10,11). It was hypothesized that both oxidative muscles, myocardium and soleus, likely express few and identical proteins capable of exerting intracellular control over mitochondrial respiration, whereas the glycolytic muscles lack these proteins (10,11). In oxidative muscle cells, these

proteins may participate in the organization of the intracellular energetic units, representing the functional and structural complexes of mitochondria with adjacent ATPases. This type of organization likely confers specific regulatory properties of respiration to these muscles (12,13). To define the nature of these proteins, we isolated genes that are expressed in common between both types of oxidative muscles but not expressed in glycolytic muscle (extensor digitorum longus). Thus, muscle cDNA libraries for soleus (S), ventricular myocardium (H), and extensor digitorum longus (E) were generated, and H was subtracted against E, resulting in a cDNA population of heart-specific genes (HE). Finally, kindred DNA amplification was carried out to obtain common cDNA fragments from HE and S, and the shared genes (SHE) were confirmed by Southern analysis (14). This is a rapid method to isolate candidate genes for organization of intracellular energy production in oxidative muscle cells.

MATERIALS AND METHODS

Animals

The adult white laboratory male mice were used as the source of differ-

ent muscle tissues. The animals were kept, fed, and studied in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Oligonucleotides and Adapters

All the oligonucleotides and adapters used (Table 1) were ordered from Genset (Paris, France), except those produced by BD Biosciences Clontech (Palo Alto, CA, USA).

Total RNA Preparation

The total RNA was isolated from the ventricular myocardium, soleus, and extensor digitorum longus of the mouse using total RNA isolating kit (Macherey-Nagel, Düren, Germany). All procedures were performed according to the manufacturer's protocol. The amount of total RNA isolated varied from 100 to 500 ng, depending on the type and mass of muscle obtained from one mouse. The isolated RNAs were ethanol-precipitated and resuspended in water in a final concentration of 10 ng/ μ L.

Total cDNA Synthesis

The total RNA was reverse-transcribed to obtain the total cDNA using the SMARTTM cDNA synthesis protocol (15–17). The first step was the mixing of 4 μ L respective total RNA sample (10 ng/ μ L), 2 μ L 10 μ M SMART oligonucleotide, and 2 μ L 10 μ M SMART CDS primer. The mixture was heated at 70°C for 2 min. Thereafter, the following additions were made: 4 μ L 5 $\times$ first-strand buffer (Invitrogen, Carlsbad, CA, USA), 2 μ L 20 mM DTT, 2 μ L 10 mM dNTP, 2 μ L SuperScriptTM II reverse transcriptase (Invitrogen), and 200 U/ μ L ribonuclease inhibitor (MBI Fermentas, Vilnius, Lithuania). The resulting mixture was heated at 42°C for 2 h, followed by the addition of 80 μ L TE buffer.

The second step was generation of total double-stranded cDNA from the total single-stranded cDNA obtained from the procedures in step one. It was started with mixing of 10 μ L of the step-one mixture with 10 μ L 10 $\times$ Ad-

vantage 2TM PCR Buffer (BD Biosciences Clontech), 2 μ L 10 mM dNTP, 2 μ L 10 mM 5'-PCR Primer (Table 1), 2 μ L Advantage 2 Polymerase Mix (BD Biosciences Clontech), and 74 μ L water. PCR was performed within the following parameters: 30 cycles of 95°C for 30 s, 65°C for 30 s, and 68°C for 6 min, and a final extension at 68°C for 9 min. Starting from cycle no. 18, 10 μ L of the reaction mixture were removed after every third cycle and analyzed on a 2% agarose/SYBR Green[®] gel (Molecular Probes, Eugene, OR, USA) to find out the optimal number of PCR cycles. After that, this PCR regime was applied to all muscle samples studied. The resulting total cDNA was purified using NucleoSpin[®] Extract Columns (Macherey-Nagel), ethanol-precipitated, and resuspended in water at a concentration of 50 ng/ μ L.

Subtractive Hybridization, PCR Amplification, and Removal of the Nested Primer's Sequences from the Ends of cDNA Fragments of HE Population

Tester preparation. Here, the first step was the restriction cleavage of total double-stranded cDNA obtained from the myocardium (H). Thus, 8 μ L of respective total cDNA (50 ng/ μ L), 1 μ L *Rsa*I restriction enzyme (10 U/ μ L; New England Biolabs, Beverly, MA, USA, or MBI Fermentas), and 1 μ L respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 h. Then, the mixture was purified using NucleoSpin Extract Columns, and cDNA fragments were ethanol-precipitated and resuspended in water at a concentration of 50 ng/ μ L. In a second step, the adapters were added to two separate reaction mediums, both containing 2 μ L 50 ng/ μ L digested cDNA, 1 μ L T4 DNA ligase (New England Biolabs or MBI Fermentas), 1 μ L T4 Ligase Buffer (respective supplier), and 4 μ L water, but in the presence of 2 μ L 10 μ M adapter 1 in one and adapter 2R (10 μ M) (Table 1) in the other. The reactions were incubated overnight at 16°C. The enzyme and excess adapters were removed by using NucleoSpin Extract Columns, and the tester cDNAs were ethanol-precipitated and resuspended in water at concentrations of 10

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ng/ μ L. Thus, two different tester preparations from the same cDNA fragment, one with adapter 1 and another with adapter 2R, were obtained.

Driver preparation. The driver was prepared from the cDNA of extensor digitorum longus similar to the tester preparation, except the resulting driver fragments were resuspended in water at concentrations of 200 ng/ μ L and the adapters were not ligated to the ends of cDNA.

Subtractive hybridization. Driver dsDNA (1.5 μ L; 300 ng) was added to a pair of Eppendorf® tubes containing 1 μ L hybridization buffer [200 mM HEPES, pH 8.3, 2 M NaCl, 0.1 mM EDTA, 10% (w/v) PEG 8000] and 15 ng adapter 1-ligated tester cDNA in one tube and 15 ng adapter 2R-ligated tester cDNA in the other, mixed, overlaid with mineral oil, denatured for 5 min at 95°C, and hybridized for 10 h at 68°C. These two samples were then pooled and mixed, freshly denatured (5 min at 95°C) driver dsDNA (200 ng) in 2 μ L hybridization buffer was added, and the reaction (second hybridization) was incubated for 10 h at 68°C. Thereafter, the final hybridization mixture was diluted 10-fold in TE and stored at -20°C.

PCR amplification. The first step was accomplished in a 25- μ L reaction mixture containing 1 μ L diluted, subtracted cDNA, 1 μ L 10 μ M PCR primer 1, and 23 μ L PCR master mixture prepared using Advantage 2 Polymerase Mix, according to the manufacturer's instructions. PCR was performed using the following parameters: initial incubation at 75°C for 5 min, then 27 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min, and a final extension at 68°C for 5 min. The amplified products were then diluted 10-fold in water and amplified in 25 μ L of the reaction mixture containing 1 μ L diluted amplified cDNA, 1 μ L 10 μ M nested PCR primer 1, 1 μ L 10 μ M nested PCR primer 2R (Table 1), and 22 μ L PCR master mixture. PCR amplification was performed for 12 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, with a final extension step at 68°C for 5 min. The PCR products were analyzed on an agarose/SYBR Green gel. Then, the mixture was purified using NucleoSpin Extract Columns, and cDNA fragments were ethanol-precipitated and re-

suspended in water at a concentration of 50 ng/ μ L, resulting in an HE population of cDNA.

Removal of the nested primer's sequences from the ends of the DNA fragments of HE population. Because the DNA fragments of HE population of cDNA generated by subtractive hybridization had different primer (nested primer 1 or nested primer 2R; Table 1) sequences on both ends, it was necessary to remove those sequences before the addition of adapters during the kindred DNA amplification procedure (see below). The sequences were removed as follows: 8 μ L 50 ng/ μ L HE cDNA, 1 μ L 10 U/ μ L *RsaI* restriction enzyme, and 1 μ L respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 h. Then, the mixture was purified using NucleoSpin Extract Columns, and the cDNA fragments were ethanol-precipitated and resuspended in water at a concentration of 50 ng/ μ L. The efficiency of removal of primers 1 and 2R from the ends of the HE fragments was monitored by PCR amplification in a 25- μ L reaction mixture containing 1 μ L diluted (10 $\times$) *RsaI*-cleaved HE, 1 μ L 10 μ M primer 1, 1 μ L 10 μ M primer 2R, and 22 μ L PCR master mixture. The PCR amplification was performed as follows: 94°C for 5 min, 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, and the final extension at 68°C for 5 min. When the PCR products were analyzed by the agarose/SYBR Green gel electrophoresis, no visible PCR products were observed, indicating that the nested primer sequences were totally removed from HE.

Kindred DNA Amplification

Phase 1 (Figure 1): cDNA preparation. The total cDNA of soleus was restriction-digested exactly as for the cDNA of myocardium (H) described above. The adapters were then added to cDNA of soleus cleaved with *RsaI*, as described above. The excess enzyme and adapters were removed by using NucleoSpin Extract Columns, and the DNA was ethanol-precipitated and resuspended in water at a concentration of 10 ng/ μ L. Thus, two different DNA preparations were obtained, one (soleus) with the adapter 1 and another

(HE) with the adapter 2R at the end of the DNA fragments.

Phase 2: hybridization of S with HE. The hybridization solution was generated by mixing 1.5 μ L adapter-ligated cDNA (S and HE, each 15 ng) with 1 μ L 4 $\times$ hybridization buffer, prepared according to the PCR-Select cDNA Subtraction Kit instructions (BD Biosciences Clontech). The solution was overlaid with mineral oil, denatured for 5 min at 95°C, and allowed to hybridize overnight for 8 h at 68°C. The final hybridization was then diluted 10-fold in TE and stored at -20°C.

Phase 3: Filling in the end of adapters and PCR amplification. The filling in of adapters takes place in the initial incubation needed to prepare the PCR template. For kindred DNA amplification, the two-step procedure of PCR amplification was performed exactly as described for the PCR amplification step above. The PCR products obtained were considered to be the SHE fraction containing the cDNA fragments in common between the myocardium (H) and soleus (S) were purified using the NucleoSpin Extract Columns, eluted in 50 μ L water, and analyzed by agarose/SYBR Green gel electrophoresis.

Cloning of the Kindred DNA Amplification cDNAs

The kindred DNA amplification products were cloned by using pTZ57R/T plasmid vector within the InsT/aclone PCR Product Cloning Kit according to the manufacturer's protocol (MBI Fermentas). The host strain used was JM109.

Preparation of DNA for Southern Hybridization

The cloned DNA inserts were amplified by PCR directly from the bacterial colonies. Bacterial cells of a given colony were transferred into 25 μ L of the reaction mixture containing 1 μ L 10 μ M nested PCR primer 1, 1 μ L 10 μ M nested PCR primer 2R (Table 1), and 23 μ L PCR master mixture. PCR amplification was performed by 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, and the PCR products were purified using NucleoSpin Extract Columns in an elution volume

of 50 µL and analyzed by agarose/SYBR Green gel electrophoresis.

Dot-Blot Hybridization

Five microliters of amplified cDNA fragments were directly blotted onto a

nylon filter. All the processes—DNA labeling, hybridization, and detection—were performed using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions.

Phase 1. Preparation of samples.

A. Addition of adapter 1 to sample 1:

B. Addition of adapter 2R to sample 2:

Phase 2. Hybridization.

The samples are mixed, denatured and hybridized:

Phase 3. Filling of the ends of adapters and PCR amplification.

The list of possible combinations of DNA fragments after the filling of the ends of adapters:

* Only the DNA fragments that have different adapters at both ends can be exponentially amplified.

Primers:

Figure 1. Kindred DNA amplification strategy. White boxes represent restriction-digested analyzable DNA fragments. The double-labeled boxes, black combined with white and gray combined with black, represent adapter 1 and adapter 2R long strands, respectively. Black boxes also correspond to the PCR primer 1 sequence, whereas the small white and gray boxes represent the PCR primer 1 and primer 2R sequences, respectively. The smallest white and gray boxes represent adapter 1 and adapter 2R short strands, respectively (also see Table 1).

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RESULTS AND DISCUSSION

The Principles of Kindred DNA Amplification

Figure 1 shows the principles of kindred DNA amplification. In phase 1, the adapters are added to the ends of restriction-digested tester DNA fragments: adapter 1 to one population of DNA fragments (Figure 1, phase 1A) and adapter 2R to the other (Figure 1, phase 1B). In phase 2, the samples (DNA fragments with adapters) are mixed, denatured, and hybridized. As the ligation of adapters does not occur with 100% fidelity, the reaction mixture contained several probable combinations of DNA fragments, both with and without adapters (Figure 1, phase 3). The method is based on a concept that only those DNA fragments that have different adapters on its both ends can be amplified exponentially (Figure 1, phase 3). These particular fragments can be generated only if they were present in both DNA sample populations analyzed. The sense strand of the fragment with one adapter's long strand at its end hybridizes with the antisense strand, possessing the other adapter's long strand at its end. The DNA fragments present in only one sample can generate fragments during hybridization having the same adapters at both its ends. However, these fragments cannot be amplified during PCR because the long inverted repeats of the adapters form stable "panhandle-like" structures after each denaturing-annealing PCR cycle (1–3).

Application of Kindred DNA Amplification to Identify cDNA Fragments in Common Between Different Types of Oxidative Muscle

The aim of these experiments was to reveal the cDNA fragments that characterize oxidative muscle cells and also represent a pool of DNA in common between two different types of oxidative muscles—murine myocardium and soleus. Our earlier studies indicated that different types of oxidative muscle may possess a similar regulatory mechanism of mitochondrial respiration that is responsible for the low apparent affinity of mitochondria for ADP in

these muscles, whereas this mechanism seems to be absent in glycolytic muscles (10,12,13). Hence, the isolation and identification of genes responsible for muscle type-specific control of oxidative phosphorylation are required to reveal the nature of that mechanism. We determined that obtaining a large cDNA pool is preferable for isolating regulatory genes, and consequently, a new method was designed. Accordingly, the total cDNA from mouse heart was first subtracted against the total cDNA of extensor digitorum longus, a fast-twitch glycolytic muscle, resulting in the HE cDNA population. The kindred DNA amplification method was then applied to isolate the common cDNA pool between the HE and the total cDNA of soleus (Figure 1). The analysis of the products obtained (Figure 2) indicates that the cDNAs in common (SHE) became visible as two dominating bands of 200 and 250 bp, which were inserted into the pTZ57R/T vector. Altogether several thousand clones, exclusively specific to oxidative muscle, were generated. Approximately 100 clones were randomly picked and amplified. The efficacy of kindred DNA amplification was verified by Southern dot-blotting (Figure

3). In this analysis, 54 DNA fragments were randomly selected from the total set of cDNA, and three identical blots were performed. One of the membranes was hybridized with the DIG-labeled soleus total cDNA (Figure 3A) and another with DIG-labeled mouse heart total cDNA (Figure 3B). The results of those two hybridizations were similar insofar as 54 clones hybridized with cDNA probes in both cases, and two negative controls (water and *E. coli* DNA) did not hybridize. The third membrane (Figure 3C) depicts clones hybridized with DIG-labeled total cDNA of extensor digitorum longus, and they were blotted in the same order as in Figure 3, A and B. A very rare pattern of binding was observed, indicating that a large majority of genes, specific to extensor digitorum longus, were eliminated from the entire pool of cDNA in common between myocardium and soleus (SHE). The (+) sign marked in Figure 3C indicates the cDNA for G3PDH used as a positive control. This glycolytic enzyme, which exists in all muscle tissue, was used to determine whether the hybridization was successful. This signal superseded the five other spots seen on the filter. Thus, these results indicate that the

Figure 2. Analysis of kindred DNA amplification by 2% agarose gel electrophoresis. The dominating bands of cDNAs in common (SHE) between heart and soleus are visible at 200 and 250 bp (left lane). Lambda DNA/*Pst*I size marker's fragments (MBI Fermentas) are visualized in the right lane.

Figure 3. Evaluation of the common cDNA pool (SHE) by Southern hybridization. Three identical filters blotted with the SHE clones were probed with total cDNA from soleus (A), heart (B), or extensor digitorum longus (C). The dots indicate binding of labeled cDNA of different sources to complementary cDNA sequences of the SHE pool. (-) and (+) represent the negative and positive controls, respectively.

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common cDNA pool (SHE) obtained by kindred DNA amplification did not contain the cDNA fragments characteristic of extensor digitorum longus and, as such, represented a set of genes specific for slow-twitch oxidative muscle.

In conclusion, the method described here serves as a reliable and practical tool for cloning genes in common from different tissues. This method permitted us to rapidly isolate candidate genes involved in muscle type-dependent control of mitochondrial respiration in oxidative muscle. The function of these genes remains to be defined in future studies.

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Ulo Puurand, Lumme Kadaja, and Enn K. Seppet
*University of Tartu
Tartu, Estonia*