Although the heating step is postulated to increase SAGE cloning efficiency (5), a small percentage of lower molecular weight DNA still existed in the purified concatemers (Figure 4), resulting in low cloning efficiency. To better remove lower molecular weight DNA from the concatemer mixture, we substituted a QIAquick PCR purification filter column for the heating step. Incorporation of the column in concatemer purification results in a significant loss of contaminating low molecular weight DNA and, as a result, improves cloning efficiency (Figure 4 and Table 2). For convenience, DNA inserts cloned in pUC19 were sequenced with M13 Forward primer only. A single automated sequencing run can yield 600–1000 nucleotides of high-quality DNA sequence—equivalent to 45–70 SAGE tags.

We have demonstrated that the combined use of modified primers for ditag PCR amplification and the incorporation of a filter column purification step for concatemer separation reduced the required number of reactions and enhanced the efficiency of SAGE library construction. These modifications substantially improve the performance of SAGE. The modifications described here may also be applicable to LongSAGE methodology (15).

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Size-selection of cDNA libraries for the cloning of cDNAs after suppression subtractive hybridization

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Here we describe the establishment of size-selected cDNA libraries for the cloning of full-length cDNAs that were initially identified by suppression subtractive hybridization (SSH) technology as being differentially expressed. First, the SSH-cDNA fragments were used as ^{32}P-probes to verify their level and differential pattern of expression by virtual Northern and to establish their corresponding full-length cDNA size. Second, cDNAs were separated by size on agarose gels and used to construct size-selected cDNA plasmid libraries, which were then screened by colony hybridization with the SSH-cDNA fragments. We conclude that the described approach complements SSH technology by allowing efficient cloning and characterization of the corresponding full-length cDNA from any desired cell type or species. This approach will give researchers the ability to specifically target and study differentially expressed genes in an efficient manner for functional genomic studies.

INTRODUCTION

Suppression subtractive hybridization (SSH) is a powerful technique to identify genes that are differentially expressed from one group of cells or tissue compared with another (1,2). The SSH approach necessitates the digestion of the cDNAs with a four-base cutter restriction enzyme (*Rsa*I). This cDNA digestion is necessary to allow equivalent amplification by PCR of small and large cDNA fragments. Amplification by PCR is achieved by ligating adaptors to both ends of digested cDNAs. Hence, SSH allows the identification, by sequencing and BLAST analysis in GenBank® databases, of differentially expressed genes as cDNA fragments that average 0.2–1 kb in size (2). However, the partial cDNA sequences compared by BLAST in GenBank databases may not adequately identify the orthologous gene. This is especially true for species not included in large-scale genomic sequencing projects where nucleotide sequence databases are incomplete (3,4). Various reasons may explain the inability to identify the corresponding gene: (*i*) the transcript represents a novel gene not yet isolated in other species; (*ii*) a different part of the transcript is present in GenBank; (*iii*) alternative splicing of the same gene in different species; or (*iv*) the sequence has diverged to the extent that it is not recognized as an orthologous sequence (5). Therefore, the use of SSH may necessitate the cloning of the corresponding full-length cDNAs to identify the gene of interest with confidence or to permit the characterization of new genes. Moreover, obtaining the full-length cDNA may allow the production of the corresponding recombinant protein and the identification of mRNA splice variants. The isolation of full-length cDNAs by conventional screening of complex cDNA libraries constructed in phage or in plasmid vectors (6) is time-consuming and expensive, and in some cases may not be successful. This paper presents a technical approach to limit the complexity of a cDNA library by size-selecting cDNAs generated by the SMART™ cDNA synthesis method (7). The described approach allows the cloning of full-length cDNAs that were initially identified by SSH as being differentially expressed. Thus, the objective was to establish and validate the screening of size-selected cDNA libraries with cDNA fragments derived from a gene expression profiling experiment to allow the cloning of corresponding full-length cDNAs.

MATERIALS AND METHODS

Sample Collection, SSH, and Virtual Northern Blots

Granulosa cells (GCs) were collected from bovine ovarian follicles at specific stages of development [growing dominant follicles (DOM: >8 mm) or ovulatory hCG-induced follicles (OVU: >11 mm)], and total RNA was isolated and quantified as previously described (8). Gene expression in GCs from follicle groups was compared using SSH. To generate sufficient amounts of double-stranded cDNAs for an SSH experiment, both DOM and OVU cDNAs were amplified using the SMART PCR cDNA synthesis kit (BD Biosciences Clontech, Mississauga, ON, Canada) (7,9). The OVU cDNAs were subtracted against DOM cDNAs (forward reaction: OVU-DOM) or vice versa (reverse reaction: DOM-OVU) using PCR-Select™ cDNA subtraction technology (BD Biosciences Clontech) (1,2). The screening of the OVU-DOM and DOM-OVU subtracted cDNA libraries showed that 40% (379/950) and 24.2% (228/940) of the cDNA clones were differentially expressed, respectively. The cDNA clones identified as differentially expressed in the SSH experiment were used to determine the size of their corresponding full-length cDNA and to confirm their differential expression pattern by virtual Northern blot analysis (7,10). To perform virtual Northern blots, SMART PCR cDNA synthesis technology was used to generate cDNAs from 1 µg total RNA isolated from GCs of different-sized follicles. For each GC sample, 10 µL of the cDNA products generated from the secondary PCR were separated on 0.8% TBE-agarose gels, for 4–5 h at 80–90 V (6) in parallel with molecular weight standards (1-kb ladder, ϕX174-RF/*Hae*III and λ/*Hin*dIII; Amersham Biosciences, Baie d'Urfe, QC, Cana-

da). The gel was washed in TBE buffer for 30 min, and cDNA samples were transferred onto nylon membrane (Hybond®-N^+; Amersham Biosciences) by alkaline capillary transfer (6) and cross-linked by UV treatment (150 mJ; Gs Gene Linker, Bio-Rad Laboratories, Mississauga, ON, Canada). The SSH cDNA clones that were identified as differentially expressed between GCs of different follicular groups were produced by PCR amplification as described in the PCR-Select differential screening kit and then purified by separation on a 1% TAE-agarose gel. The corresponding cDNA bands were extracted from the gel (QIAquick™ Gel Extraction Kit; Qiagen, Mississauga, ON, Canada) and quantified. The cDNAs (75 ng) were labeled with [^{32}P]dCTP by random priming (Megaprime™ DNA Labeling System; Amersham Biosciences), and the probes were purified (QIAquick Nucleotide Removal kit; Qiagen), heat-denatured, and used to hybridize virtual Northern membranes under conditions described below.

Establishment of Size-Selected cDNA Libraries

One microgram of total RNA was reverse-transcribed using the SMART PCR cDNA synthesis kit in a 10-µL reaction volume with oligo$(dT)_{30}$ [CDS: 5′-AAGCAGTGGTAACAACGCAG-AGTACT$_{(30)}$(A/C/G/T)(A/G/C)-3′], 42 ng T4 gene 32 protein (Roche Applied Science, Laval, PQ, Canada) (11) and PowerScript to generate the first-strand cDNA. Second cDNA strands were produced with the SMART II 5′-anchored oligonucleotide (5′-AAGCAGTGGTA-ACAACGCAGAGTACGCGGG-3′) and PCR-amplified for 15 cycles using Advantage™ 2 DNA polymerase (BD Biosciences Clontech). The cDNA pool was diluted to 50 µL with TE buffer. One microliter of the cDNA pool was used in a secondary 100-µL reaction for 18 cycles using the PCR primer 5′-AAGCAGTGGTAACAACGCAGAG-T-3′ and Advantage 2 DNA polymerase. The cDNA products (10–30 µL) of the secondary PCR along with molecular weight standards (1-kb ladder, ϕX174-RF/*Hae*III and λ/*Hin*dIII) were separated on 0.8% TBE-agarose gels for 4–5 h at 80–90 V (6). Fragments (3–5 mm) of the agarose gel were excised at the desired molecular weights as determined by initial virtual Northern blot analyses, and the cDNAs were purified using the QIAquick Gel Extraction Kit and eluted in 30 µL TE. To generate sufficient cDNAs to establish the size-selected cDNA libraries, the purified cDNAs were amplified in 50-µL reactions. Mixtures of 2 µL purified cDNAs and 1 µL 10 µM PCR primer in a total volume of 25 µL were covered by mineral oil and heat-denatured for 10 min at 95°C and then transferred to ice. Twenty-five microliters of a mixture including 5 µL 10× Advantage 2 PCR buffer, 1 µL 50× dNTP mixture (10 mM each), and 1 µL enzyme mixture were added to each cDNA/primer mixture. The reaction consisted of a denaturation step at 95°C for 1 min, followed by 12–14 cycles at 95°C for 5 s, 65°C for 5 s, and 68°C for 6 min. The cDNAs were purified using the QIAquick kit, followed by a verification of the size and the quality of the amplified cDNAs by separating a 5- to 10-µL volume of the size-selected samples on a 1% TAE-agarose gel and observation under UV illumination, and then transferred onto Hybond-N^+ nylon membranes. These transferred cDNAs served as positive controls during the screening step of the size-selected cDNA libraries as described below. The remainders of the purified size-selected cDNA samples were cloned into the pDrive plasmid (Qiagen PCR cloning kit) in 10-µL ligation reactions: 4 µL (500 ng) purified cDNAs, 1 µL plasmid (50 ng), 5 µL ligation master mixture, and incubated at 14°C for 16 h. Two microliters of each ligation reaction were used to transform 50 µL competent TOP10F′ bacteria (BD Biosciences Clontech) by heat shock treatment (12). The transformed bacteria were incubated in 1 mL SOC medium for 45 min with shaking, and 100 µL of these final cultures were plated onto 100-mm S-Gal/LB agar plates (Sigma-Aldrich, St. Louis, MO, USA) with 40 µg/mL kanamycin and grown overnight at 37°C.

To array the size-selected plasmid cDNA libraries, white colonies were individually picked with sterile toothpicks, used to inoculate 96-well plates containing 200 µL/well LB media supplemented with 8.8% glycerol, 55 mM K_2HPO_4, 1 mM $MgSO_4$, 26 mM KH_2PO_4, 15 mM $NH_4(SO_4)$, and 40 µg/mL kanamycin, and were grown for 14 h at 37°C without shaking. Bacteria were transferred from 96-well plates with a 96-well pin replicator (Nalge Nunc International, Rochester, NY, USA) onto positively charged nylon membranes (Pall Biodyne B Nylon Membrane; Nalge Nunc International) overlaid onto rectangular LB agar plates (Plate Omnitray; Nalge Nunc International) with 40 µg/mL kanamycin. Multiple 96-well plates (4–9 plates) were transferred onto each nylon sheet, thereby reducing the number of nylon membranes to hybridize. The transferred bacterial colonies were grown for 10 h at 37°C and then kept at 4°C, whereas the master 96-well plates were frozen at -70°C. Pen marks were made on the nylon membranes for each row and column to allow proper alignment of the bacterial colonies grown on the nylon membranes with the original 96-well plate. Bacterial colonies were denatured by alkali treatment (6), and the released DNA was cross-linked to the membranes by 150 mJ UV irradiation (Gs Gene Linker).

Screening of the Size-Selected cDNA Library

The cDNA probes used to screen the size-selected cDNA libraries were derived from the SSH experiment. The probes were labeled with ^{32}P by random priming as described above. The membranes were incubated in a prehybridization solution (600 mM NaCl, 120 mM Tris, 4 mM EDTA, 0.1% Na_4pyrophosphate, 0.2% SDS, and 500 µg/mL heparin) (13) for 4 h at 68°C and then overnight in the same solution with the purified ^{32}P-DNA probe. For probes containing a poly(A) tail, 10 µL of 60 µM poly(dA)pdT$_{12-18}$ (Amersham Biosciences) were heat-denatured and added in the prehybridization and hybridization solutions. Membranes were washed for 20 min in 2× SSC, 0.1% SDS at 68°C, followed by two 1-h washes in 0.1× SSC, 0.1% SDS at 68°C. Membranes were exposed to phosphor screen for 4 h to overnight, and the images were digitized (Storm™ 840; Amersham Biosciences). Replicates of

the pen marks on the hybridization membranes were made on transparencies and aligned with the printed digitized images to allow the identification of positive clones. The positive clones were then characterized by inoculating overnight bacterial cultures from the frozen 96-well master plates, followed by plasmid miniprep purification (QIAprep®; Qiagen), digestion by *Eco*RI, and analysis on 1% TAE-agarose gel to verify the size of the insert. Sequencing of the cDNA insert was then performed as previously described (8).

RESULTS AND DISCUSSION

The application of SSH has permitted us to identify genes (cDNA fragments) that are differentially expressed in GCs of growing dominant versus hCG-induced ovulatory ovarian follicles (data not shown). We first verified if these cDNA fragments were truly differentially expressed and determined the size of their corresponding full-length cDNAs. Virtual Northern analysis demonstrated that gene 1 (3.5 kb in size) and gene 2 (5 kb) were selectively expressed in GCs of hCG-induced ovulatory follicles when compared to GCs of dominant follicles (Figure 1). Conversely, gene 3 (2.1 kb) was selectively expressed in GCs of dominant follicles when compared with GCs of hCG-induced ovulatory follicles.

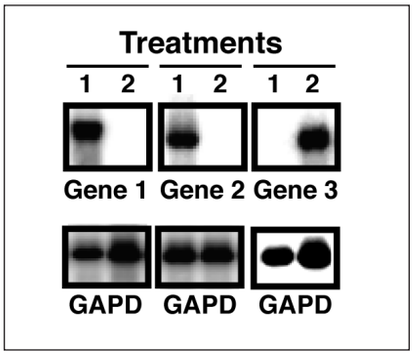

Figure 1. Virtual Northern analysis of genes identified as differentially expressed by SSH. Treatment 1 corresponds to GCs of hCG-induced ovulatory follicles, and treatment 2 to GCs of dominant nonovulatory follicles. Genes 1 (3.5 kb in size) and 2 (5 kb) are selectively expressed in GCs of hCG-induced ovulatory follicles, whereas gene 3 (2.1 kb) is selectively expressed in GCs of dominant follicles. GAPDH was used as a control gene.

To characterize the corresponding full-length cDNAs, since SSH generates only cDNA fragments, SMART cDNAs were separated by size using agarose gel electrophoresis (Figure 2A) and cut into 3- to 5-mm fragments. The agarose fragments corresponding to the desired cDNAs to be cloned, as determined by initial virtual Northern analyses, were extracted and re-amplified by an optimized number of PCR cycles. Re-amplification was necessary to generate sufficient amounts of the size-selected cDNAs to perform ligation reactions into the pDrive plasmid vector. The number of PCR cycles to be used was optimized for each extracted and size-selected cDNA pool by visualizing the PCR products of increasing numbers of cycles (ranging from 12 to 15; Figure 2B) on agarose gels. The number of PCR cycles chosen was that which generated the highest signal intensity of the desired molecular weight cDNA compared to background. We observed that increasing number of PCR cycles beyond the optimized number could result in increased background products that appear as lower or higher molecular

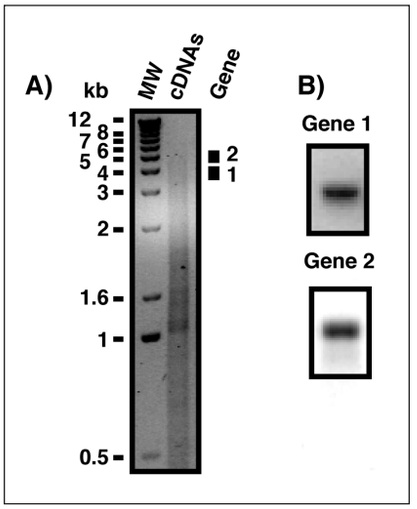

Figure 2. Generation of full-length and size-selected cDNA library. (A) The SMART cDNA products (10–30 µL) were separated on 0.8% TBE-agarose gels for 4–5 h at 80–90 V. Gel fragments of 3–5 mm (size-selected as shown by the corresponding bars on the right side) were excised, and the cDNAs were extracted from the agarose. (B) The size-selected cDNAs were re-amplified for an optimized number of PCR cycles (12–14) in a 50-µL reaction volume. PCR product (5–10 µL) was visualized on agarose gel (gene 1, 3.5 kb; gene 2, 5 kb) before cloning into the pDrive vector.

weight products compared to the selected cDNA size. Although rapid and convenient, it should be noted that the use of PCR for cloning purposes may introduce point mutations in the cDNA to be characterized, even if a mixture of DNA polymerase including a proofreading DNA polymerase and a limited number of PCR cycles are used.

The size-selected cDNAs were cloned into the pDrive plasmid vector, the proportion of bacterial colonies harboring a recombinant plasmid was high (>98%), and the colonies were well separated at the described plating dilution. The number of bacterial colonies to transfer to 96-well plates depended on the signal intensity observed by the virtual Northern analysis. There appeared to be a qualitative relationship between the intensity of the virtual Northern signal and the percentage of positive colonies, but that relationship was not quantified. Following the transfer of individual bacterial colonies into 96-well plates, these colonies were grown for 16 h and then transferred onto nylon membranes with a 96-well pin replicator and incubated for another 10 h to generate well-formed colonies. Plasmids contained in these bacteria were released by alkali treatment and bound to the nylon membrane.

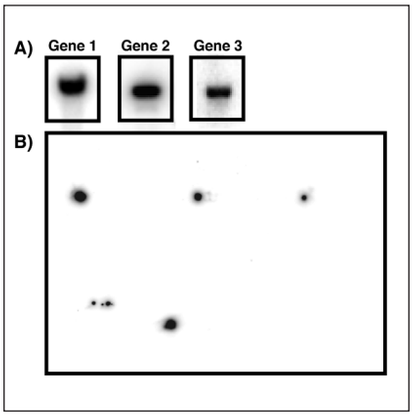

Figure 3. Screening of the size-selected cDNA library. (A) The size-selected cDNA PCR products (Figure 2B) served as Southern blotting positive hybridization controls during the screening of the size-selected cDNA libraries. (B) Colony hybridization screening of 384 colonies (4 × 96-well plates/nylon membrane) of a size-selected cDNA library (4.5–5.5 kb) for gene 2. The nylon membranes were hybridized with a ^{32}P-labeled probe derived from a differentially expressed cDNA fragment (760 bp) identified during an initial SSH experiment.

Probes used to screen the size-selected cDNA libraries were derived from the SSH experiment. The cDNAs that were re-amplified and analyzed on agarose gels (Figure 2B) to generate the size-selected cDNA libraries were transferred to nylon membranes for use in positive-control Southern blotting experiments performed in tandem with the hybridization of the size-selected cDNA libraries. Absence of hybridization signal for the positive control meant that the size-selected cDNA gel fragment was not selected or amplified by PCR properly during construction of the size-selected cDNA library. Conversely, a hybridization signal for the positive control without hybridization signal of the size-selected cDNA library meant that an insufficient number of bacterial colonies were screened, thereby necessitating a second round of screening.

Hybridization screening results of different size-selected cDNA libraries varied from 2.1% positive colonies for gene 1 (4 colonies out of 192 screened) to 0.13% for gene 3 (2 colonies out of 1536 screened). The results of the establishment and screening of three different size-selected cDNA libraries (genes 1–3) showed that the technique is applicable to different sizes of cDNAs, and the number of positive colonies obtained following hybridization screening is independent of the size of the cDNA to be characterized. The identification of positive colonies following the hybridization step was obvious given the stronger specific hybridization signal compared with negative colonies. Analysis of the positive colonies showed no false positives following the screening of the three different size-selected cDNA libraries. Restriction digest analysis followed by sequencing of cDNA inserts showed that they contained the full-length cDNAs corresponding to the cDNA probe fragment derived from the SSH experiment.

These results provide evidence that the establishment of size-selected cDNA libraries derived from SMART cDNA synthesis allows efficient cloning of full-length cDNAs from the corresponding differentially expressed cDNA fragments (>200 bp) obtained from SSH experiments. The described technical approach is valuable, complements SSH technology (2), and could also be used to clone the corresponding full-length cDNAs of differentially expressed cDNA fragments identified by mRNA differential display RT-PCR (14). The described approach presents an advantage over the RecA-based cloning technique in that it does not require the synthesis of cDNA-specific oligonucleotides to allow enrichment and/or cloning of the full-length cDNA (9). Moreover, the advantage over 5′- and 3′-rapid amplification of cDNA ends (RACE) (6) is also evident because RACE only allows PCR amplification of cDNA fragments, requires the synthesis of gene specific oligonucleotides, and often does not yield consistent or reliable results. Indeed, the base content of some SSH-derived cDNA fragments may not permit the design of efficient PCR primers. We conclude that the size-selected cDNA library approach complements SSH technology by allowing efficient cloning and characterization of the desired full-length cDNAs from any cell type or species. This approach will provide researchers with the ability to target and study specific differentially expressed genes in an efficient manner for functional genomic studies.

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Dictyostelium discoideum transformation by oscillating electric field electroporation

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Dictyostelium discoideum *has been used as a genetically tractable model organism to study many biological phenomena. High-efficiency transformation is a prerequisite for successful genetic screens such as mutant complementation, identification of suppressor genes, or insertional mutagenesis. Although exponential decay electroporation is the standard transformation technique for* D. discoideum*, its efficiency is relatively low and its reproducibility is weak. Here we optimized the oscillating electroporation technique for* D. discoideum *transformation and compared it to the exponential decay electroporation. A 20-fold increase in the efficiency was reproducibly achieved. This alternative electroporation technique should facilitate future genetic approaches in* D. discoideum.

INTRODUCTION

The social amoeba *Dictyostelium discoideum* lives in the soil as a unicellular organism feeding on bacteria. Upon starvation, *Dictyostelium* amoebae initiate a development program that leads to the formation of a multicellular organism and fruiting bodies containing spores (1). With a haploid genome of 34 Mb, *D. discoideum* represents a powerful genetic system to investigate many biological processes such as cell motility and development (2), phagocytosis and endocytosis (3), and host-pathogen interaction (4–7). Therefore, the availability of an efficient transformation technique is desirable to perform genetic screens, such as cDNA suppressor isolation or insertional mutagenesis, or to generate mutants by homologous recombination. Exponential decay electroporation has been used to introduce DNA molecules into *D. discoideum* cells for more than a decade (8). A significant increase in the transformation efficiency would help researchers and bring *D. discoideum* genetics to its full potential. A recent study demonstrated that *D. discoideum*

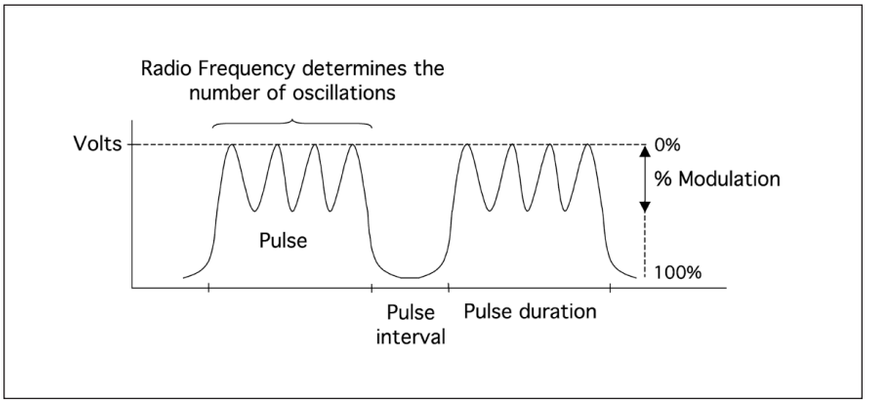

Figure 1. Scheme of the oscillating electric field electroporation. The electric field is applied as a succession of pulses. A rectangular pulse is obtained when modulation is set to 0%, and an oscillating pulse when modulation is set to 100%. Different parameters can be regulated: voltage, pulse duration, number of pulses, RF, pulse-interval duration, and percent of modulation.