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Construction and Sequence Analysis of Subtraction Complementary DNA Libraries from Human Preimplantation Embryos

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Purpose: *Because stage-specific genetic expression in human preimplantation development is not sufficiently studied, we have undertaken the construction of a subtraction complementary DNA (cDNA) library enriched for transcripts specific for human blastocysts.*
Methods: *For this purpose individual pools of cDNAs synthesized from four hatched blastocysts and three cleaving 8- to 10-cell embryos were exposed to suppression subtractive hybridization to minimize the presence of transcripts of housekeeping genes and other genes of maternal origin known to be expressed earlier in preimplantation development. Random clones of this library were sequenced and analyzed using the BLAST algorithm.*
Results: *The resulting subtraction library had a complexity of 3×10^5 and an average size of inserts of about 0.8 kb. Sequencing of random library clones revealed the following human genes: CD9 antigen, fatty acid binding protein, ferritin heavy chain, amiloid precursor, MAP kinase messenger RNAs, DNA clone127H14, messenger RNA for diacylglycerol kinase, a sequence homologous to C1 inhibitor, messenger RNA for the KIAA0145 gene, and others.*
Conclusions: *The presence of these genes in human preimplantation development suggests expression specific to the blastocyst stage.*

KEY WORDS: preimplantation development; blastocyst; subtraction cDNA library; random clone sequencing; stage specific gene expression.

INTRODUCTION

Polymerase chain reaction (PCR)-based complementary DNA (cDNA) libraries from individual human blastocysts were recently constructed to investigate genetic expression in human preimplantation development (1–4). Similar work has been recently performed in the mouse model, resulting in sequencing and characterization of gene expression profiles for mouse blastocysts (5). We have previously reported the presence of β-actin, CD-59, homeobox OCT-3, histone 3.1, human ribosomal protein S25, two houskeeping genes, hexokinase 1 and serin/threonin phosphorylase, and also four other expressed sequence tags (ESTs) (2,3) previously detected in fetal brain and pancreatic tumor cells (6). However, stage-specific gene expression patterns in preimplantation development were not studied. It is known that oocyte RNAs may persist as long as the early blastocyst stage (7), and many zygotic transcripts from the cleavage stage can be found also in blastocyst. At the same time, it is obvious that with the differentiation of different cell types in blastocyst, there should be changes in gene expression. To identify a stage-specific expression profile for human blastocysts, we undertook the construction of a subtraction cDNA library enriched in transcripts specific for human blastocysts, demonstrating the presence of 26 known and 29 novel genes in the blastocyst stage.

MATERIALS AND METHODS

Human preimplantation embryos were obtained from in vitro fertilization patients at Illinois Masonic Medical Center, Chicago, IL, with informed consent approved by the institutional review board of this institution. These embryos would otherwise have been discarded for various reasons, described earlier (2,3).

Primers for reverse transcription and polymerase chain reaction (PCR) were synthesized using an

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Applied Biosystems 381A DNA Synthesizer (Camden, NJ). The compatibility of primers, the absence of hairpins, and primer–dimer formation were analyzed using the OLIGO program software (8).

We tested samples for contamination with genomic DNA or nonhuman nucleic acids, using nested PCR systems able to detect single molecules of DNA (9). Using single cumulus cells as a positive control, 3 μl of each sample after reverse transcription was first amplified with primers designed for nucleotide sequence of the CFTR intron (cystic fibrosis transmembrane regulator gene), to exclude any detectable molecules of genomic DNA. Then the samples were exposed to nested PCR with a primer system designed for a DNA fragment of *Neurospora crassa* (accession number of the corresponding gene frequency, L14465); this microorganism is present in dust and provides an excellent test for the purity of the laboratory environment. Genomic DNA from human blood out of hood was used as a positive control in these experiments, showing that none of the embryos used was contaminated with either genomic DNA or DNA of *N. crassa*.

To synthesize cDNA we used a modified method described previously (2,3). During cDNA amplification two rounds of PCR were performed. The first round consisted of 25 cycles and was performed using a hot start to prevent annealing of the rest of the random primer Univ-N_6. The amplified cDNA was enriched to a high molecular fraction by chromatography on a Sephadex G-50 Medium (Pharmacia, Piscataway, NJ) minicolumn (10). Second round PCR was performed with the primers Lyb-3 and Univ (Fig. 1).

To construct the subtractive cDNA library we used a modified method described by Diatchenko *et al.* (11). cDNAs from cleaving embryos (driver cDNA) and blastocysts (tester cDNA) were treated with nuclease S1 and purified on chromatography columns. Ten nanograms of the tester and 600 ng of the driver cDNA were used for subtractive library construction (11) (Figs. 2a and b). The resulting subtracted cDNA was then used for phage λ library construction.

The protocols of phage packaging, propagation, and analysis were published earlier (2,3). Random clones were sequenced and analyzed using the BLAST algorithm.

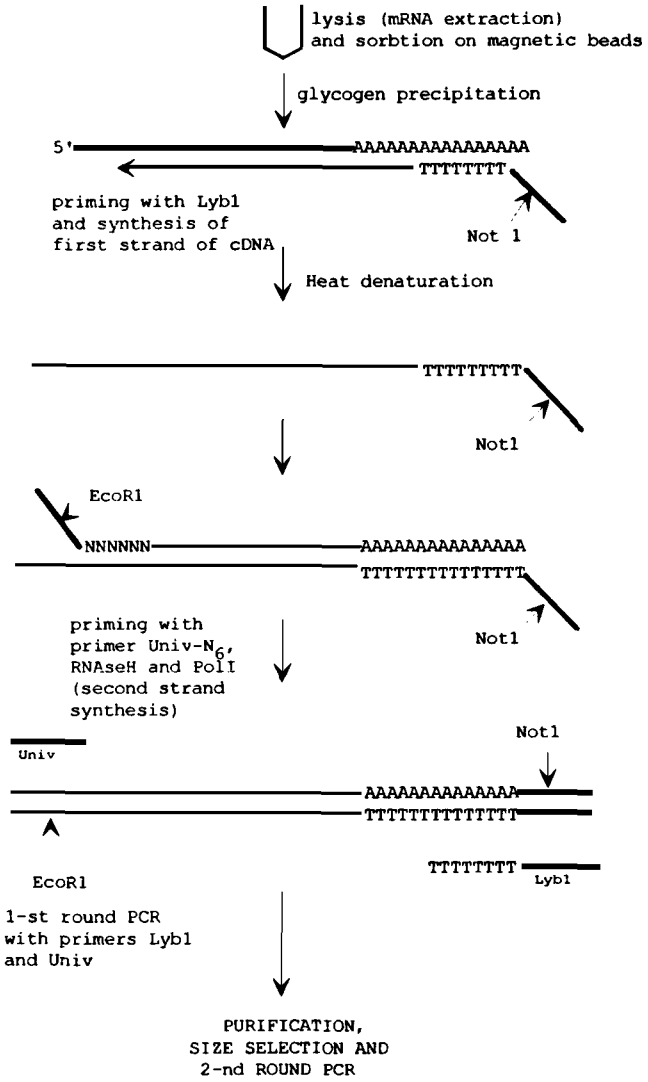

Fig. 1. Principal steps in the construction of cDNA libraries.

RESULTS AND DISCUSSION

The resulting subtraction library had a complexity of 3×10^5 and an average insert size of approximately 0.8 kb. Approximately 5% of the clones contained no inserts and 3% contained inserts of 50 bp or less. Random clones of this library were single-pass sequenced and analyzed. We classified the sequences into three categories, depending on the sequence identity. One of them consisted of known genes, represented by sequences of 98% or more identity to known gene sequences. Possible PCR errors in four rounds of the PCR used for library construction may explain the 2% mismatches in some of these cases. As shown in Table 1, some of these sequences belong to well-characterized genes, while the others show no identity with definite genes, but correspond to nucleotide sequences cloned in cosmids and mapped on human chromosomes. Seven of the 14 genes presented in Table I, α-tubulin, surface antigen CD9, elongation

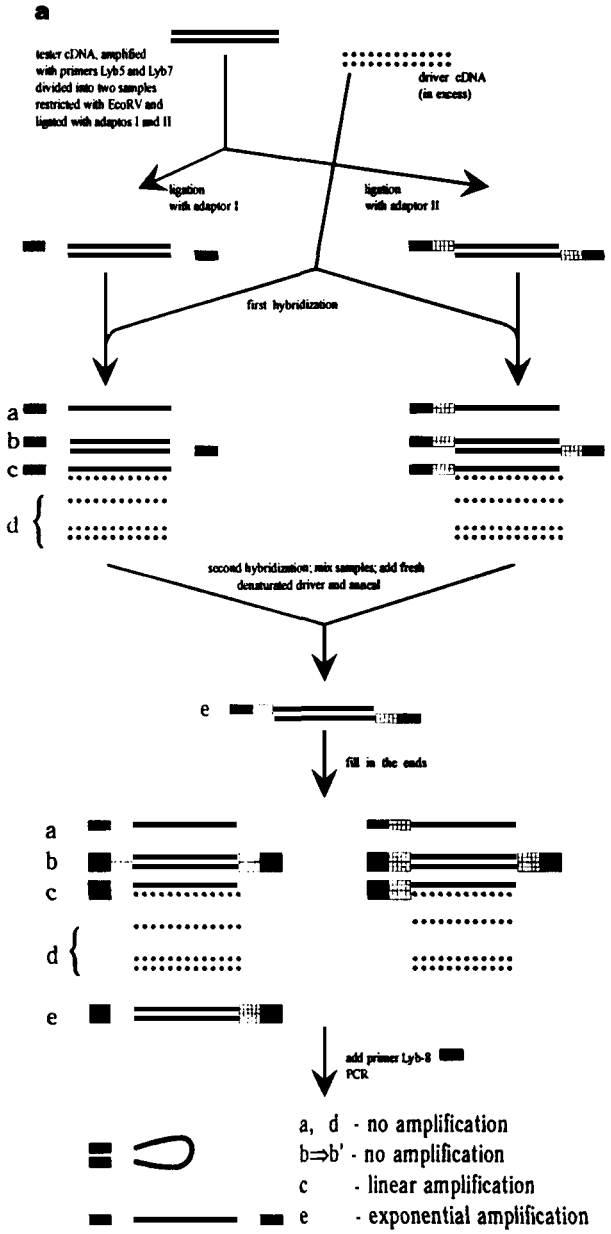

Fig. 2. Schematic diagram of PCR-select cDNA subtraction [11]. (a) Steps of construction. Black boxes represent the outer part of the adaptors' longer strands and correspond to PCR primer Lyb-8. Open boxes represent the inner part of adaptor I and correspond to the nested primer Lyb-9. Hatched boxes represent the inner part of adaptor II and correspond to the nested Lyb-10. (b) Adaptors and primers used for construction of normalized and subtracted cDNA libraries.

factors EF-1-α and EF-1-γ, vacuolar proton adenosine triphosphatase, fatty acid binding protein, and integrin α-3, were previously demonstrated to be expressed in mouse preimplantation development (5). The other group of sequences showed 85%–97% identity with

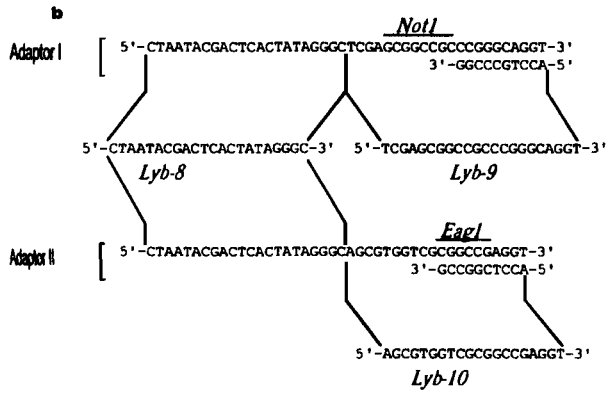

Fig. 2. Continued.

known genes, such as for amyloid precursor, human messenger RNA homologous to mouse p21, human fatty acid binding protein, and human ferritin heavy chain. Finally, 47% of the sequences represented novel or unknown genes, requiring further analysis for their identification (Fig. 3).

Data presented are the first description of a stage-specific genetic expression library obtained using subtraction cDNA libraries from human preimplantation embryos. The genes detected for integrin α-3 chain and laminin receptor are involved in the control of cell adhesion and may be related to blastocyst implantation. In fact, expression of the laminin receptor was previously reported in the mouse blastocyst (5), while integrin α-3 chain was shown in both mouse (12) and human (13). Some of the genes listed in Table I are related to the regulation of cell division, including

Table I. Genes Found in the Subtraction Library

Clone	Most homologous sequence	Percentage identity
SLB83	Adenylsuccinate lyase	100
SLB4	MAP kinase	100
SLB45	Surface antigen CD9	100
SLB58	KIAA0437	100
SLB68	Elongation factor EF-1-γ	100
SLB26	Elongation factory EF-1-α	99
SLB52	α-Tubulin mRNA	99
SLB10	Diacylglycerol kinase Δ	98
SLB23	Vacuolar proton ATPase	98
SLB24	α-Enolase	98
SLB49	Integrin α-3 chain	98
SLB55	RAN-1 mRNA	98
SLB74	Human mRNA for laminin receptor	98
SLB27	Human CD24 signal transducer mRNA	96
SLB54	Human fatty acid binding protein	94
SLB1	Amyloid precursor	92
SLB33	Human mRNA homologous to mouse p21	86
SLB60	Human ferritin heavy chain	86

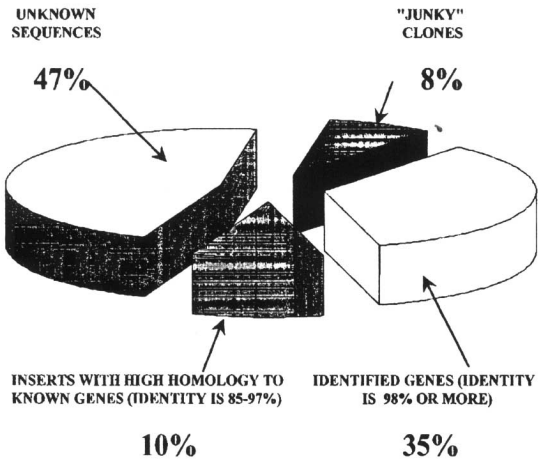

Fig. 3. Pie diagram of the sequences found in the subtracted library.

RAN-1, involved in DNA replication and onset of and exit from mitosis (14), and MAP (mitogen-activated protein) kinase, involved in cell cycle regulation (15). The other sequence identical to human messenger RNA (accession number X64899) homologous to mouse p21 is involved in cyclin/cyclin-dependent kinase system regulation (16). Finally, the CD24 gene, detected in our experiments, controls signal transducer mRNA and is involved in B-cell activation (17). It is of interest that the expression of another B cell–related gene, B-cell receptor–associated protein 32, was reported in the mouse blastocyst (5). The subtraction process did not eliminate all housekeeping genes, and some were still present in the library, probably due to amplification of partial lengths of tester and driver PCR products. Although we were able to enrich the library with stage-specific genes, to achieve a more complete subtraction, representation of the tester and driver cDNAs might be increased by using a larger quantity of embryos for subtraction experiments.

The results presented show that subtraction cDNA library construction is a feasible and efficient technique for the analysis of stage-specific genetic expression in preimplantation development.

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