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RESEARCH

Construction and Characterization of a Goat Mammary Gland cDNA Library

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Abstract A lactating goat mammary gland cDNA library was constructed by using a modified commercially available cDNA library construction kit protocol. The resulting clones were sequenced and functionally analyzed through cross-species genomic comparison to assess (1) the capacity and functional quality of the constructed library for subsequent research and (2) the efficiency of the procedural modifications. The study resulted in the construction of a high-quality mammary gland cDNA library, which was characterized by (1) the total recombinants number of 1.4×10^7 colony-forming units (cfus) that was at least 10 times greater than the number expected from the application of the standard kit protocol, (2) the recombinants rate of 96%, and (3) the average insert size of 1,082 bp. BLAST analysis of sequenced clones against GenBank databases determined 55.7% of clone redundancy, 22 known function gene clusters, and 29 novel gene clusters. The analysis of the primary gene expression profile showed that 59% of the tested clones were genes that coded for milk proteins while 16% of the clones coded for ribosomal, metabolism, immune response, and translation proteins. The remaining 25% of the tested clones were described as novel genes. Cross-species comparison showed that 77% of characterized gene clusters were successfully identified by using resources from other ruminants and unrelated species. This outcome is in consonance with the common belief that the genomic resources that have been generated across species are potentially powerful tools that could be used for enhancing the molecular understanding of less genomically studied species, such as goat.

Keywords cDNA library · Magnetic particle · Mammary gland

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Introduction

Goat milk is the most widely consumed by humans worldwide, among milk from other animal species [1]. Although its nutritional value is similar to that of cow milk, goat milk has higher digestibility of protein and fat, distinct alkalinity, higher buffering capacity, hypo-allergenicity, and higher concentration of short and medium chain length fatty acids [2–5]. Goat milk has been recommended as a hypo-allergenic infant food or cow milk substitute for people who suffered from eczema, asthma, chronic catarrh, colitis, hayfever, epigastric distress, and abdominal pain caused by allergic reactions to cow milk [6, 7]. Also, its therapeutic values in medical treatments of malabsorption and stomach ulcer has been reported [8, 9].

Despite its benefits, millions of people worldwide do not like goat milk and its by-products primarily because of its peculiar "goaty" flavor or smell [10–12]. With current genomic advances such limitations could be corrected through gene manipulations [13–15]. However, because of the insufficiency of genomic resources and information pertaining to goat, it is very difficult to improve this species, in general, or solve the repulsive goaty smell problem, in particular, using modern technologies. Our recent survey (as of September 19, 2007) showed that while there were

2,244,051 and 577,146 nucleotide sequences available for cow and sheep, respectively, there were only 18,890 nucleotide sequences for goat in the NCBI nucleotide database. Of these there were 5,120, 13,506, and 264 goat records on core nucleotide, Expressed Sequence Tags (ESTs), and genome survey sequences (GSS), respectively. This is insignificant, when compared to 229,309, 1,517,987, and 496,755, for cow, and 12,843, 187,701, and 376,602, for sheep, for the three parameters, respectively. Such tremendous genomic resources development and research effort gap among the three ruminants underscores the importance of the present research. The primary focus of this study was to enhance the development of goat genomic resources and information through the construction, sequencing and characterization of mammary gland cDNA library of lactating Chinese Xinong Saanen dairy goat. The results could be used to expand genomic research activities on this species.

Materials and Methods

Tissue Sample Collection

Samples of mammary tissue were collected from a healthy lactating Xinong Saanen dairy goat, a breed of *Capra hircus*, raised at Xinong Saanen Goat Breeding Farm of Northwest A&F University. After local anesthesia, the mammary tissues (1–2 g) were surgically removed from the body by veterinarians and promptly frozen in liquid nitrogen. The tissue samples were stored at −80°C until they were used for RNA isolation.

Total RNA Isolation and mRNA Purification

Frozen mammary tissues (2 g) were homogenized in liquid nitrogen using mortars and pestles. Total RNA was extracted using Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instruction. RNA quality was assessed by ultraviolet absorbance at 260 and 280 nm wavelength and electrophoresis. mRNA was purified from total RNA by using PolyA Ttract mRNA Isolation System II (Promega, Madison, WI, USA) according to the vendor's instructions.

Construction of cDNA Library

The primary cDNA library was constructed from purified mRNA by using SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning kit (Invitrogen, Carlsbad, CA, USA) with some modifications. The first strand cDNA was primed by a biotinylated (B-) oligo $(dT)_{15}$ primer containing an internal *Not* I restriction site of the following sequence (B-)GAC(B-)TAG(B-)T(B-)$TCTAGATCGCGAGCGGCCGCCCT(T)_{15}$. The initial reaction mixture included 1 μg of purified mRNA, 1 μg of biotinylated oligo dT primer, and RNase free water for the total volume of 20 μl. The reaction mixture was pre-heated at 70°C for 10 min and then reduced to 45°C prior to adding 7 μl of 5× first strand buffer, 4 μl of 0.1 M DTT, 2 μl of 10 mM dNTP mix, and 2 μl of SuperScript™ II reverse transcriptase (200 U/μl). The first strand reaction was then incubated at 45°C for 65 min. The second strand cDNA was synthesized by adding a combination of 30 μl of 5× second strand buffer, 4 μl of 10 mM dNTP mix, 1 μl of *E. coli* RNase H (2 U/μl), 4.2 μl of *E. coli* DNA polymerase I (10 U/μl), 1.2 μl of *E. coli* DNA ligase (10 U/μl), and RNase free water up to 115 μl, to the first strand reaction mixture and then incubated at 16°C for 2 h. After blunting the double strand cDNA ends with T4 DNA polymerase followed the Invitrogen kit's protocol, the cDNAs were concentrated by mixing with pre-washed Streptavidin-Paramagnetic Particles (SA-PMPs) and washed according to the vendor's instruction (same as the protocol of PolyA Ttract mRNA Isolation System (Promega, Madison, WI, USA)). A *Sal* I adapter from the Invitrogen kit was then ligated to SA-PMPs bound cDNAs under the condition recommended by the manufacturer. Adaptor ligated cDNAs were then immobilized through magnetic rack and cleaned up with a series of washes, as it was done in previous steps. A *Not* I restriction digestion using the reagents and the protocol supplied with Invitrogen kit was performed thereafter to release immobilized cDNAs from the SA-PMPs. The released cDNAs were subjected to 1% low melting point agarose (LMA) gel electrophoresis for size fractionation. The cDNAs of 500 bp and above were recovered from LMA gel by melting excised gel block at 65°C, followed by gel phenol (60% phenol, 10 mM NaCl, 10 mM Tris–HCl) extraction and ethanol precipitation. Purified cDNAs were quantified via electrophoresis or spectrophotometer, prior to its directional cloning into *Not* I–*Sal* I-cut pCMVSPORT6 vector and electroporating into ElectroMAX DH10B competent cells, following the Invitrogen kit's instruction.

Library Quality Determination

Several simple experiments were conducted to check the quality of the constructed goat mammary cDNA library. Regular plating assays with series dilution (10^{-2}, 10^{-3}, and 10^{-4}) were performed to determine the titer and total capacity of the library. Because the traditional blue/white positive selection system for recombinant transformants

was not applied in this modified protocol, we subjected 23 randomly picked colonies from preceding referred plates to enzymatic restriction analysis using *Sal* I and *Not* I restriction enzymes followed by electrophoretic analysis. The recombinant efficiency was determined by the number of colonies that contained visible inserts over the total number of tested colonies. For the constructed cDNA library to be considered for downstream application, it had to demonstrate a recombinant efficiency of at least 96%. The amplified total cDNA library mixture was also subjected to *Sal* I and *Not* I enzymatic restrict digestion and electrophoretic analysis for determining library clone distribution pattern.

cDNA Sequencing and Sequence Analysis

Randomly picked 115 individual clones were subjected to 5′-end single-pass DNA sequencing by automated sequencer ABI PRISM 3730 with BigDye terminator v3.1 (Applied Biosystems, USA) and M13r sequencing primer (AGCGGATAACAATTTCACACAGG). Raw sequence data were manipulated to remove vector and terminal sequence of low reliability [16]. The cDNA sequences were then analyzed with BLASTN from NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/) against GenBank Nucleotides collection (nr/nt) and Expressed Sequence Tags (EST) databases, and were clustered into individual gene groups. The sequence clustering was determined manually by the similarity percentage of aligned sequences. If two aligned sequences were more than 90% identical, while the offset of the poly (A) site was below 25 nucleotides, then the two sequences were described as belonging to the same group or cluster [17]. Further, a single best quality sequence was selected as the representative of each cluster and subjected to cross-species comparative analysis. On the basis of the sequence match percentage, similarities, scores and *E*-value, individual clusters were assigned potential gene functions.

Results

The Capacity and Quality of Primary Mammary Gland cDNA Library

A directionally cloned, high-quality cDNA library was successfully constructed for EST sequencing and clone collection. The results showed that the library was enriched with 1.4×10^7 colony-forming units (cfus) of 1.9×10^6 cfus/ml titer, which were determined by regular plating assay. The library's recombinant rate was 96%, which resulted from an estimate of 22 recombinants out of 23 randomly picked colonies. The recombinant average insert size was 1,082 bp, and the length of the majority of inserts was over 500 bp (Fig. 1). The analysis of clone distribution pattern along with the undigested original library mixture showed that the clone size ranged from 0.5 to 5.0 kb, and the average size of most abundant genes was about 1,000 bp (Fig. 2).

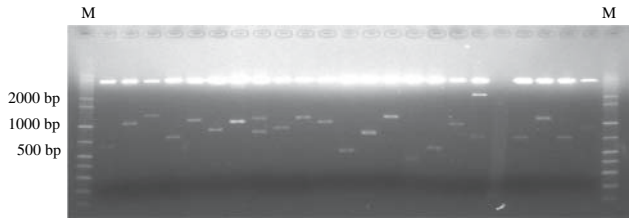

Fig. 1 Determination of library recombinants rate and average insert size by restriction analysis of 23 randomly selected clones. (M: 1 kb plus DNA ladder)

Sequencing Results and Data Analysis

The analysis of sequencing data showed that the primary cDNA library contained 55.7% of redundant clones. All

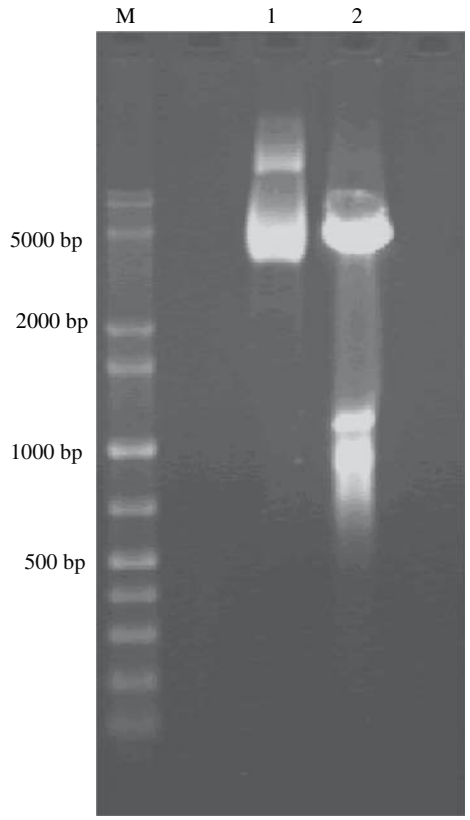

Fig. 2 Clone distribution pattern of goat mammary cDNA library. (M: 1 kb plus DNA ladder. Lane 1: undigested library. Lane2: *Not* I/ *Sal* I digested library

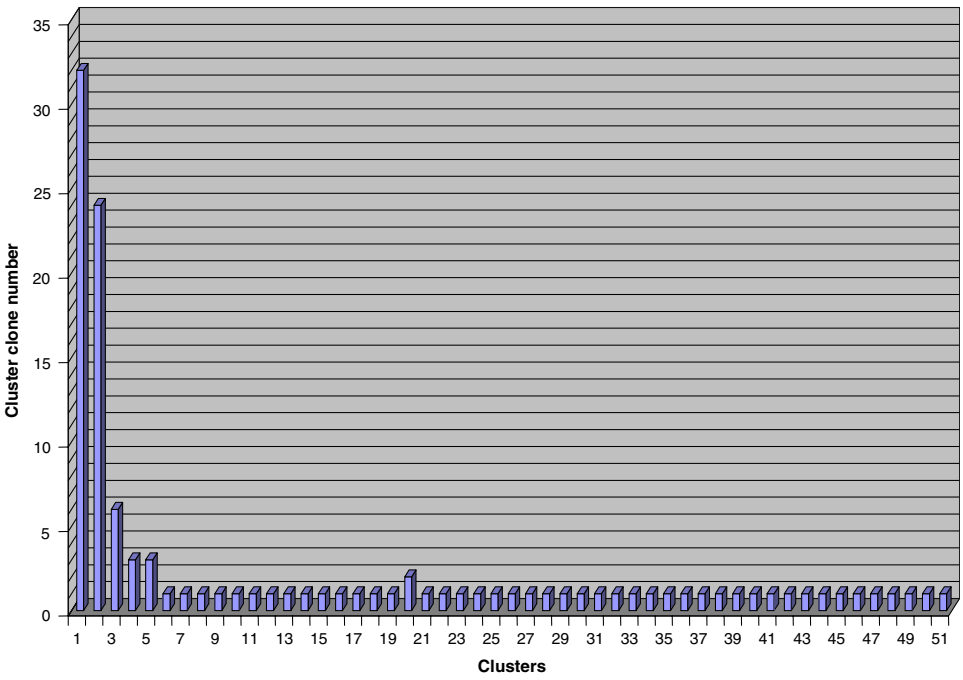

Fig. 3 Sequence redundancy of goat mammary cDNA library

sequenced clones were categorized into 51 gene clusters, which included 45 singletons (88%), four clusters of 2, 3, 3, and 6 clone copies (8%), and two clusters of 24 and 32 clone copies (4%), respectively (Fig. 3). The BLASTN results (Fig. 4) showed that 22 of 51 gene clusters (43.2%) representing 86 clones matched known functions of previously described genes, whereas the additional 29 gene clusters (56.8%) representing the remaining 29 clones had no matches either within goat or across species, or had matched other sequences of unknown functions (Table 1). The latter were, therefore, identified as novel genes. Further analysis showed that amongst the 22 gene clusters with known functions, 5 clusters (22.7%), which represented 44 clones, had matched goat genes; whereas 3 (13.6%), 11 (50%), and 3 (13.6%) other gene clusters, which represented 28, 11, and 3 clones, had matched sheep, cow, and human genes, respectively.

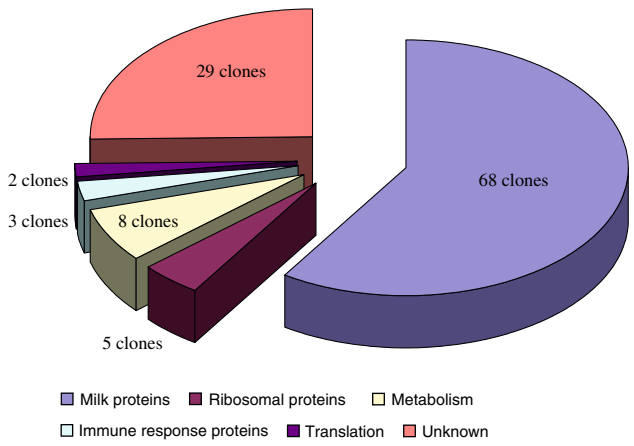

Fig. 4 Clone sequence functional categorization of goat mammary cDNA library

Detailed analysis of known function sequenced clones revealed that they coded for a wide spectrum of proteins, including milk, ribosomal, metabolic, immune response, and translational proteins (Fig. 4). Of all the sequenced clones, 68 coded for milk proteins, amongst which 32 clones coded for beta-lactoglobulin, whereas 24, 6, 3, and 3 additional clones coded for beta-casein, αs2-casein, prealpha-lactalbumin, and kappa-casein, respectively. Except beta- and kappa-casein, all other milk proteins, in addition to glycosylation-dependent cell adhesion molecule-1 (2 clones) and fatty acid synthase (1 clone), matched known goat functional genes (Table 1). Additional 5 single copies of unrelated clones coded for ribosomal proteins, which included S2, S6, L21, S18, and S10 proteins. Another eight unrelated single clone sequences coded for different proteins. The latter were each involved in distinct metabolic processes of 2 different molecules for lipids, energy and amino acid, and 1 molecule for fatty acid and nucleic acid. Amongst the remaining 5 clones, 3 coded for two types of immune response proteins while 2 others coded for two individual translational proteins (data not shown).

Discussion

Modified cDNA Construction Procedure

The construction of this high-quality primary cDNA library is an essential step in future wide spectrum of goat mammary gland genomic research. It could be used for, but not

Table 1 Sequence analysis of known function genes

Clone ID	Cluster number	Query length (bp)	Matched percentage[a]	Homolog genes	Score	*E*-value	Similarity (%)[b]
1112230(M3)	1	549	97.6	*C. hircus* beta-lactoglobulin mRNA	1130	0.0	99
1112228(M1)	2	598	99.0	Sheep mRNA for beta-casein	1122	0.0	99
1112257(M30)	3	524	97.7	*C. hircus* mRNA for as2-casein	1172	0.0	100
1112241(M14)	4	373	98.9	*Ovis aries* kappa-casein (CSN3), mRNA	708	0.0	98
1112281(M55)	5	553	99.5	Goat mRNA for prealpha-lactalbumin	1358	0.0	99
1112242(M15)	6	598	97.7	*Bos taurus* ribosomal protein S2 (RPS2), mRNA	1637	0.0	97
1112235(M8)	7	535	97.6	*B. taurus* ribosomal protein S6, mRNA	1187	0.0	97
1112247(M20)	8	542	87.3	Homo sapiens ribosomal protein L21 (RPL21), mRNA	729	0.0	93
1130597(M128)	9	520	96.7	*B. taurus* ribosomal protein S18 (RPS18), mRNA	896	0.0	96
1112336(M111)	10	544	96.1	*B. taurus* ribosomal protein S10 (RPS10), mRNA	977	0.0	97
1112250(M23)	11	439	93.4	*B. taurus* NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9 kDa (NDUFA4), mRNA	745	0.0	97
1112341(M116)	12	556	96.6	*B. taurus* NADH dehydrogenase (ubiquinone) Fe–S protein 7, 20 kDa, mRNA	1261	0.0	96
1112272(M46)	13	650	88.6	Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3, mRNA	585	8E-164	88
1112322(M97)	14	581	98.6	*B. taurus* ornithine decarboxylase antizyme 1-like (LOC506973), mRNA	1247	0.0	98
1112326(M101)	15	553	92.2	*B. taurus* serum amyloid A 3, mRNA (cDNA clone MGC:133567 IMAGE:8057366), complete cds length = 591	553	0.0	92
1121185(M10)	16	487	94.5	*B. taurus* mammary serum amyloid A3.2 precursor, mRNA, complete cds	779	0.0	95
1130606(M124)	17	243	97.9	*C. hircus* fatty acid synthase (FASN) mRNA, complete cds length = 8,114	466	2e-128	99
1112254(M27)	18	470	97.9	Homo sapiens small EDRK-rich factor 2, mRNA (cDNA clone MGC:8965 IMAGE:3902770), complete cds	846	0.0	97
1112299(M74)	19	536	97.2	*O. aries* mRNA for Ig gamma 2 constant region heavy chain	1045	0.0	97
1112277(M51)	20	631	99.5	*C. hircus* mRNA for glycosylation-dependent cell adhesion molecule-1 (GLYCAM1 gene)	1233	0.0	99
1112323(M98)	21	521	96.4	*B. taurus* eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), mRNA	983	0.0	99
1112332(M107)	22	563	99.5	*B. taurus* tumor protein, translationally controlled 1, mRNA	1588	0.0	99

[a] Matched percentage: aligned target sequence length (bp) over query sequence length (bp)

[b] Similarity (%): matched target sequence length (bp) over aligned query sequence length (bp)

limited to, gene sequencing for structural studies, molecular markers and gene screening, and gene expression. By applying the standard protocol we used, one can generate a cDNA library of approximately 1.0×10^6 cfus. When this original protocol was adjusted as previously described in the procedure, we were able to construct a much improved quality and capacity cDNA library of 1.4×10^7 cfus, 96% recombinants efficiency rate, and greater than 1 kb average insert size. One of the prominent benefits was highlighted by Soares et al. [18], who through experimental analysis had concluded that such great cDNA library capacity and quality, which had been constructed from any tissue, is more likely to represent all expressed genes 'including the rarest ones'. The present cDNA library's capacity is at least 10 times greater than what could have otherwise been achieved without the original kit protocol modifications. This proves that by integrating magnetic particle technology into the primary cDNA library construction, we had enhanced the original protocol efficiency by preventing artificial losses of synthesized cDNAs which otherwise could have occurred during the library construction [19]. Such modifications have potential benefits, primarily to research labs or projects with limited sample tissues or mRNAs. It could also be beneficial to novice scientists with

less experience in the application of cDNA library construction, as well as to experienced scientists in the field that could save their time and labor while improving the quality of their results.

We also applied electrophoresis cDNA size selection in substitution of column chromatography technology, which requires the measurement of radioactivity of each eluted fraction and complicated calculations [20]. By eliminating radioisotope from the protocol, we not only eliminated related complex calculations, but also enhanced safety for both lab scientists and working environment. This modified protocol also enables a direct visualization of cDNA on electrophoresis for more accurate size selection.

Characterization of Primary Mammary Gland cDNA Library

Using bioinformatics techniques, sequencing data of randomly selected clones were analyzed for developing a primary gene functional profile and assessing the overall functional quality of the primary cDNA library that was constructed with modified standard kit protocol. It was encouraging that observed redundancy of the library clones (55.7%) was situated within the normal range (50–65%), which had been reported earlier by Bonaldo et al. [21]. A high-novel gene discovery rate (25%) of the library had encouraged its downstream applications that led to the discovery of key genes associated with goat lactation (data not shown). Further analysis showed that the majority of known functional clones (59%) represented genes that code for milk related proteins. Such observation was expected considering the lactating stage of mammary gland sample tissues [22]. Functional profile also revealed the presence of genes coding for various metabolic and immune response proteins, which could be justified for their potential roles in milk digestion, nutrients absorption, and immunity development in baby animals [1, 2, 4, 11, 13, 14]. Milk immune response in human, for instance, has been proven to provide passive protection to the young [23]. Colostrums in cow have also been proven to offer passive immunity for the newborn calf via the intestinal absorption of intact immunoglobulins [24]. In mice, some forms of αs1-caseins had provided protection against lethal infection of *Candida albicans* by stimulating both phagocytosis and immune response [25]. Noticeably, cross-species comparison analysis demonstrated that the potential functions of about 77% of characterized gene clusters were successfully identified by using resources primarily from other ruminants such as sheep and cow, and unrelated species such as human, which is a good indication that genomic resources which had been generated across species are potential powerful tools that could be used for enhancing the molecular understanding of this less genomically studied species.

Conclusion

This article reports the use of a modified standard kit protocol to construct a primary cDNA library for goat lactating mammary gland. The modifications are simple, practical, efficient, and more importantly reproducible. It enabled us to improve the original protocol's expected cDNA library capacity by 10-fold, with a great recombinants rate (96%). The visualization of cDNA molecules via electrophoresis improved the selection of inserts of size greater than 1 kb. The functional quality of the resulting cDNA library was best reflected through its significantly biased representation of genes mostly associated with lactating stage of the mammary gland.

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