STUDY ON CONSTRUCTION OF cDNA LIBRARIES FROM RAT NORMAL LIVER AND REGENERATION LIVER WITH SMART TECHNIQUE

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ABSTRACT

The aim of the study is to construct cDNA libraries from the normal liver and regeneration liver of rat by SMART (switching mechanism at 5' end of RNA transcript) technique and analyze their quality. The total RNA was separated from the normal liver and regeneration liver of rat and the first-strand cDNA was synthesized through reverse transcription by a modified oligo (dT) primer (contained *sfi* IB site) while the SMART oligonucleotide (contained *sfi* IA site) was utilized as a template so that the first-strand cDNA could be extended over the 5' end of mRNA. The double-strand cDNA was amplified by LD-PCR (long-distance PCR) with the above two primers and then digested by *sfi* I (IA & IB) restriction enzyme. After cDNA size fractionation through Chroma Spin 400 column, the double-strand cDNA was ligated into the *sfi* I-digested lambda TriplEx2 vector and then the recombinant DNA was packaged *in vitro*. The unamplified rat normal liver cDNA library consists of 1.3×10^7 pfu/ml, and regeneration liver cDNA library consists of 1.6×10^7 pfu/ml in which the percentage of recombinant clones both are about 100%. Through testing, the high quality cDNA libraries containing full-length cDNA of rat normal liver and regeneration liver have been constructed. The titer of the amplified cDNA library is 4.5×10^{10} pfu/ml and 3.6×10^{10} pfu/ml. the average exogenous inserts of the recombinants both are about 1.5 kb. These results show that the normal liver and regeneration liver of rat cDNA libraries both have an excellent quality and lay solid foundation to study liver functions and the mechanism of liver regeneration.

KEY WORDS

cDNA library, SMART, rat, LD-PCR, liver regeneration.

INTRODUCTION

Liver is the only organ that possesses the great capability of regeneration in mammals (1). Today, the known functions of liver have been beyond imagination (2). Since Higgins and Anderson established the model of partial hepatectomy (PH) in 1931 (3), it has been widely used to study the reconstructing and regeneration of tissues and organs, cellular multiplication, dedifferentiation, stress response and the regulation of physiology

and biochemistry. Most studies about liver regeneration focus on observing the changes in cellular morphology, structure, physiology, biochemistry, metabolism, separation and function of liver regeneration factors (4). However, liver regeneration is a complicated process. It should be the result operated by many factors, and there are many genes expressed or restrained during liver regeneration (5). But how to initiate the regeneration of hepatocytes, which genes are key to liver regeneration, how to obtain full-length cDNA of these genes to study their functions is still a question? To solve them, we construct the cDNA libraries from the normal liver and regeneration liver recovering after 72 hours with SMART technique, because studies show that it is a key phase to recover 72 hours after PH (6). It lay solid foundation to study liver functions and the mechanism of liver regeneration. From the two libraries, we have obtained several up-regulated expressed genes.

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MATERIAL AND METHODS

Material

Sprague-Dawley rats (weighing 200 g - 250 g) were provided by the experimental animal house of College of Life Science in Henan Normal University. RNAase kit procured from Qiagen company, SMART cDNA library construction kit from Clontech company and package protein of λ phage from Stratagene company. Other chemicals and reagents all were of analytical grade.

Preparation of samples

According to the methods of Higgins, 2/3 of the liver of rats was dissected, then the rats were made to recover for 72 hours. Isolation of total RNA was carried out according to the protocol of RNAase kit. The quantity and integrity of total RNA was detected by ultraviolet spectrometer and by electrophoresis on a denaturing formaldehyde agarose gel stained with EtBr (ethidium bromide) (7).

Primers of constructing cDNA library

Oligonucleotide: 5'-AAGCAGTGGTATCAA CGCAGAGTGGCCATTACGGCCGGG-3'; CDS $\square$ /3'PCR primer: 5'-ATTCTAGAGGCCGAG GCGGCC GACATG-d(T)₃₀N1N-3'; 5' PCR primer: 5'-AAGC AGTGGTATCGGCGC AGAGT-3'.

Generating a cDNA library

According to the protocol of SMART cDNA library construction kit and package protein of λ phage, firstly, first strand cDNA was composed using reverse transcriptase by CDS $\square$ /3'PCR primer contained *sfi* IB site (a modified oligo (dT) primer), while the SMART oligonucleotide (contained *sfi* IA site) was utilized as a template so that the first-strand cDNA could be extended over the 5' end of mRNA. Secondly, double strands cDNA were constructed through first strand cDNA by LD-PCR (long distance PCR) with the above two primers. Thirdly, the product of LD-PCR was digested by proteinase K, then cDNA was extracted by phenol/chloroform and precipitated by ethanol and NaAc (8). Fourthly, cDNA was digested by *sfi* I (IA & IB) restriction enzyme for generating cohesive ends to link to λ phage (TriplEx2 vector). Fifthly, the fragments were selected through Chroma Spin-400 column to 16 Ep tubes, then 1.1% agarose gel electrophoresis was prepared to test the product selected, and the liquid of three lightest tubes was collected and precipitated. Sixthly, the cDNA digested by *Sfi* $\square$ was linked to two arms of λ TriplEx2 vector, then packed with package protein of λ phage *in vitro*. The unamplification cDNA

libraries were constructed.

Quality analysis of cDNA library

To calculate the titer of unamplification libraries, if the number of phage was less than $1\sim 2 \times 10^6$ pfu/ml, the linking was repeated again. After calculating the recombination fraction, the cDNA library was amplified, the titer of the amplification library was calculated. A successful cDNA library amplified should arrive to about 10^{10} pfu/ml. $\text{Pfu/ml} = (\text{number of plaques} \times \text{dilution factor} \times 10^3 \mu\text{l/ml}) / (\mu\text{l of diluted phage plated})$.

RESULTS

The quantity and integrity of total RNA

After total RNA was extracted, to measure the concentration by diluting 10 μl of total RNA into 1 ml of TE (1:100 dilution) and measured the absorbance at 260 nm and 280 nm. The OD ratio of 260 nm and 280 nm was about 2.0 (A_{260}/A_{280}). 0.8% denaturing formaldehyde agarose gel electrophoresis showed clear 28S rRNA and 18S rRNA, and the brightness rate of 28S rRNA and 18S rRNA (28S/18S) was 2:1 (Fig. 1).

Fig. 1. Denaturing agarose gel electrophoresis of total RNA from normal liver and regeneration liver
1. Normal liver 2. Regeneration liver

Construction of cDNA library

After linked to λ phage *in vitro*, packed and infected host bacteria, the titer of unamplified library from normal liver was 1.3×10^7 pfu/ml, regeneration liver was 1.7×10^7 pfu/ml, and the titer of amplified library arrived to 4.5×10^{10} pfu/ml and 3.6×10^7 pfu/ml. The results accorded with the need of constructing library. The recombination fraction was analyzed, and recombination fraction each was 99.6% and 99.8%.

PCR analysis of cDNA length in library

We randomly selected 50 clones to isolate plasmid and performed PCR with CDS $\square$ /3'PCR primer and 5'PCR primer. Results showed that all clones contained insert fragments between 200 bp and 4000 bp, and the average exogenous inserts of the recombinants each was about 1.5 kb.

Immunoscreening of cDNA library using antibody

Normal liver cDNA library and regeneration cDNA library were screened by immunoscreening with ADAMs antibody. Positive clones were obtained in two cDNA libraries. Figures showed the results of first screening (Fig. 2) and second screening (Fig. 3)

Fig. 2. Positive clones obtained from first screening of cDNA library

Fig. 3. Positive clones obtained from second screening of cDNA library

DISCUSSION

How to obtain the whole long cDNA, RT-PCR, RACE, DNA probe and immunoscreening of cDNA library were often applied (9). But it is more difficult to clone the whole cDNA by RT-PCR or RACE since unreal positive and needing fragment join each other(10). Constructing and screening cDNA library is a more effective method to catch the full-length cDNA of new protein using normal antibody by immunoscreening or screening by DNA probe (11). So the cDNA libraries from normal liver and regeneration liver of rat were constructed for studying the mechanism of liver regeneration.

The cDNA library constructed without SMART technique often brings the incompleteness of transcription, and the correct expressing product could not appear perhaps, so it is difficult to obtain full-length cDNA (12). The SMART cDNA library construction kit we used is a new product of Clontech company. The special design can make three reading frames of each insert sequence expressed, which ensure complete information (13). It is another advantage of the cDNA library we constructed to contain whole long cDNA. Normally, when mRNA was reversely transcribed into first strand cDNA, the transcription had already stopped factually before mRNA transcription was thoroughly finished (14). But in this study, by using SMART (switching mechanism at 5' end of RNA transcript) technique, the full-length mRNA could be thoroughly transcribed into cDNA.

The quantity and quality of cDNA is a key to construct high quality cDNA library (15), but the quality of cDNA is affected by mRNA, so the total RNA was isolated by RNAase kit in our study, through comparing, we thought it that more efficacious than other methods to extract RNA (7). The quantity and integrity of total RNA detected by ultraviolet spectrophotometer and by electrophoresis on a denaturing formaldehyde agarose gel was good. When performing LD-PCR, the same temperature was applied during annealing and extension, and the time of extension was delayed for obtaining integrated cDNA (16). We had screened the cDNA library using common antibody of ADAMs (a disintegrin and metalloproteinases) and caught several ADAMs related genes.

Thus, the high quality cDNA library from normal liver and regeneration liver of rat have been constructed, which made better and easy to study liver regeneration and identify objective gene in liver of rat.

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