Biologicals xxx (2015) 1–7

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Construction and characterization of yeast two-hybrid cDNA library derived from LFBK cell line

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ARTICLE INFO

Article history:
Received 7 November 2014
Accepted 28 January 2015
Available online xxx

Keywords:
FMD
Yeast two-hybrid
cDNA library
SMART

ABSTRACT

The cDNA libraries are indispensable and critical tools for performing protein–protein interaction studies. In this study, a high quality yeast two-hybrid cDNA library from the LFBK cell line was constructed and characterized. LFBK cell line was originally derived from the swine kidney cells and is highly susceptible to foot-and-mouth disease virus (FMDV) infection. The total RNA was extracted from the LFBK cells and the switching mechanism at the 5′ end of RNA template (SMART) technique was employed for the cDNA synthesis. Subsequently, double stranded cDNA was amplified by long-distance PCR, purified and co-transformed with pGADT7-rec vector in yeast strain Y187. The quality parameters of the constructed library were evaluated to qualify the constructed library. Nucleotide sequencing of the randomly selected clones from the library confirmed the swine genotype of LFBK cell line. The LFBK cDNA library was mated with the 2C protein of FMDV in yeast two-hybrid (YTH) system and several putative interaction partners were identified in the preliminary screening. The LFBK library was observed to be of high quality and could potentially be applied to protein interaction studies between FMDV and the host cells using YTH system.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of domestic livestock and several wild life species and is caused by foot-and-mouth disease virus (FMDV), a prototype of the genus *Aphthovirus* within the family *Picornaviridae* [1]. FMDV exists as seven immunologically distinct serotypes (A, O, C, Asia 1, SAT1, SAT2, and SAT3) without any cross-protection between them [2]. Even though the FMDV is the first animal virus to be discovered, the molecular intricacies of viral pathogenesis are yet to be completely understood [3]. Despite the small genome size of ~8.2 kb, FMDV proteins play plethora of roles during viral replication [4–6]. FMDV proteins interact with the host factors and exploit host cell machinery for the virus survival and replication. Thus, it is of paramount significance to study the host-virus interactions for further insights into the virus replication and pathogenesis.

The yeast two-hybrid (YTH) screens have been used widely to study interactions between host and viral proteins [7]. A well characterized, high quality cDNA library is a prerequisite and critical tool for studying host-virus protein–protein interactions using YTH system. To this end, many workers have previously reported the construction of cDNA libraries from the pathogen susceptible cell lines and tissues [8–12]. For FMDV, several susceptible continuous cell lines have been described which include BHK-21 [13], IB-RS-2 [14], IB-RS-11 [15], MVPK-1 [16] and LFBK [17]. Amongst these, LFBK cell line is a continuous cell line derived from the kidney cells of FMDV susceptible host species that survived infection with temperature sensitive mutant of FMDV C_3 [17]. Previously, LFBK cell line was thought to be derived from the calf kidney cells but the recent studies indicated the swine genotype of cell line (Personal communication, Luis L. Rodriguez, PIADC, ARS, USDA). Besides, LFBK cells have been found to be equally susceptible to all seven serotypes of FMDV and have been routinely used for various assays like plaque assay, immunofluorescent assay or plaque neutralization assay in different laboratories worldwide.

Here, we describe the construction and characterization of cDNA library derived from LFBK cells employing the switching

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http://dx.doi.org/10.1016/j.biologicals.2015.01.003
1045-1056/

mechanism at the 5′-end of the RNA transcript (SMART) technology. Using SMART technique, almost all of the mRNAs can be reverse-transcribed, even the low copy genes, thus facilitating the study of even less abundant proteins in protein interaction studies [18]. The developed cDNA library was evaluated for various parameters to qualify its application as host prey library to study interactions between FMDV and host cells using YTH system.

2. Materials and methods

2.1. Cell line and virus

LFBK cells were maintained as monolayers in Glasgow minimum essential medium (GMEM, Sigma, USA) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific, USA). The cultured cells served as the source of RNA for LFBK- cDNA library.

2.2. Isolation of total RNA and mRNA

The LFBK monolayers were trypsinized at 37 °C for 10 min to obtain individual cells. The cells were suspended in culture medium and centrifuged followed by washing of the cell pellet in PBS. The washed cell pellet thus obtained was used for the total RNA extraction using MN-Nucleospin RNA kit (Machery-Nagel, Germany) as per the manufacturer's instructions. The concentration and purity of the total RNA was determined using nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The integrity of the total RNAs was analyzed by 1% TAE-agarose gel electrophoresis. Isolation of poly(A) mRNA from total RNA was carried out using MN-NucleoTrap® mRNA kit (Machery-Nagel, Germany) according to the manufacturer's instructions. The isolated mRNA was further vacuum concentrated using Concentrator plus™ (Eppendorf, Germany). The quantity and integrity of the isolated mRNA was detected by nanodrop spectrophotometer and agarose gel electrophoresis, respectively.

2.3. cDNA synthesis

Single-stranded (ss) cDNA was synthesized using Make Your Own "Mate & Plate" Library System (Clontech, USA). The mRNA (2 μl) was reverse transcribed using SMART III oligo (5′- AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3′), CDS III primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN-3′) and SMART MMLV Reverse Transcriptase to synthesize ss cDNA. The double-stranded (ds) cDNA was amplified by long distance PCR (LD-PCR) using 2 μl of ss cDNA product and Advantage 2 PCR kit (Clontech, USA) according to the manufacturer's protocol. The PCR amplicons were analyzed using 1% agarose/EtdBr gel. Thereafter, size-fractionation of ds cDNA was carried out using CHROMA-SPIN TE-400 columns (Clontech, USA). The ds cDNA was precipitated using sodium acetate and ethanol. The quantity and quality of the purified ds cDNA was analyzed with nanodrop spectrophotometer and 1% agarose/EtdBr gel, respectively.

2.4. Construction of cDNA library

The purified ds cDNA (3.9 μg) and 3 μg of SmaI-linearized pGADT7-rec vector were co-transformed into Y187 yeast strain using lithium acetate transformation method [19] and cultured on SD/-Leu agar plates at 30 °C for 4 days. The resultant transformants were harvested in freezing medium (YPDA + 25% glycerol) and stored at −80 °C in 1 ml aliquots. The transformed cells (1 ml) were diluted in YPDA medium and 100 μl of 10^{-1} and 10^{-2} dilutions were grown on SD/-Leu plates at 30 °C for 4 days to calculate the various parameters of transformed libraries.

2.5. Evaluation of the cDNA library

The constructed cDNA library was evaluated for number of independent clones and transformation efficiency from 10^{-2} dilution SD/-Leu plates [11]. The harvested and pooled libraries were also plated on SD/-Leu plates at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions to calculate cell density, library titer and library quantity [20]. The average insert size and recombination rate of the libraries was estimated from the randomly selected 60 colonies from LFBK cDNA library. The selected colonies were subjected to PCR using Matchmaker InsertCheck PCR Mix 2 (Clontech, USA) and the products were analyzed on 1% agarose gel. Plasmids were extracted from the selected colonies using Easy Yeast Plasmid Isolation kit (Clontech, USA) and re-transformed in competent *Escherichia coli* (*E. coli*) DH5α cells by chemical transformation method [21] and plated on Luria Bertani-ampicillin (LB-Amp) agar plates. Plasmids were extracted from the positive transformants using PureLink™ Quick Plasmid MiniPrep kit (Invitrogen, USA). The extracted plasmids were subjected to nucleotide sequencing.

2.6. Nucleotide sequencing and analysis

Sixty clones were randomly selected from the LFBK cDNA library and were nucleotide sequenced with a single pass reading from the 5′end on an ABI 3130 Genetic Analyzer using Big dye terminator v3.1 (Applied Biosystems) and forward primer GBK-ADF (5′-GTAATACGACTCACTATAGGGCGA-3′). Raw sequences were manually trimmed to remove the vector and low-quality sequences. The assembled cDNA sequences were subjected to BLAST search against the nonredundant (nr) GenBank database to compare them with the currently available ESTs and genes. The BLASTn results with bit scores >80 and e-values less than 10^{-10} were considered as significant matches. The ESTs homologous to known proteins were further annotated for gene ontology (GO) terms and the GO analysis was carried out using WEB-based GEne SeT AnaLysiS Toolkit (WebGestalt) [22,23].

3. Results

3.1. Isolation and analysis of total RNA and mRNA

The $A_{260}/_{280}$ ratio and the average concentration of the isolated LFBK total RNA was found to be 2.11and 2081 ng/μl, respectively. The gel electrophoresis revealed bands of 28S and 18S rRNAs and the intensity of 28S band was twice as that of the 18S rRNA (Fig. 1) suggesting that the isolated RNA was of high quality and purity to be used for mRNA isolation. The concentration of mRNA was found to be 114.8 ng/μl (40 μl volume) and the $A_{260}/_{280}$ ratio was 2.26. The mRNA was concentrated to ~350 ng/μl using the Concentrator plus™ (Eppendorf, Germany). The gel electrophoresis revealed a smear up to ~6 kb (Fig. 2). Although some rRNA bands were faintly visible but the good length of smear reflected the quality of the extracted mRNA.

3.2. cDNA synthesis

Approximately 700 ng of mRNA (2 μl) was used for the ss cDNA synthesis using oligo-d(T) primer. The ss cDNA was further amplified by LD-PCR for ds cDNA synthesis. The optimal number of PCR cycles for ds cDNA synthesis was found to be 24. The ds cDNA smear ranged from <100 bp to 4 kb (Fig. 3a) and ~500 bp to 4 kb (Fig. 3b), before and after purification, respectively.

Please cite this article in press as: Mahajan S, et al., Construction and characterization of yeast two-hybrid cDNA library derived from LFBK cell line, Biologicals (2015), http://dx.doi.org/10.1016/j.biologicals.2015.01.003