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Rapid approach to identify an unrecognized viral agent

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Abstract

For epidemic control, rapid identification and characterization of the responsible unknown agent are crucial. To address this critical question, a method was developed for virus discovery based on a flexible nested-PCR subtraction hybridization. As a positive control, we used hepatitis C virus as a hypothetical unrecognized virus and "discover" it in the sample. Using template-switching universal long-PCR to produce large quantities of cDNA, our nested-PCR-based subtractive hybridization coupled with a single-strand deletion technology removed most of the common cDNA. Following subtraction hybridization, a cDNA library was constructed and displayed by differential reverse dot blot hybridization. This new genomic subtraction hybridization method will be ideally suited to identify rapidly any previously unrecognized viral agent.
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Keywords: Unrecognized viral agents; mRNA; cDNA; Subtraction hybridization; Long-PCR; Nested-PCR; Mung bean nuclease; cDNA library

1. Introduction

Based on past history, it is not just a hypothetical risk that many people have been infected with unrecognized viruses. By the time a new virus, such as hepatitis C virus (HCV), human immunodeficiency virus (HIV) or severe acute respiratory syndrome (SARS) associated coronavirus, is found, it is too late to save the lives of many individuals even thousands. For longer-term solutions, a powerful molecular technique is needed to rapidly identify any previously unrecognized viral agent without using virus isolation. Such a method could then be applied for investigation of future outbreaks. The most recent technologies for detecting and identifying previously unrecognized pathogens are differential display, representational difference analysis, subtractive hybridization, expression library screening, broad-range polymerase chain reaction (PCR) and serial analysis of gene expression. But they are all time-consuming and not very reproducible approaches (Allander et al., 2001).

It is now more than 10 years since the invention of differential display (Liang, 2002; Lisitsyn et al., 1993). Subtractive hybridization, a differential display-like technology, has been an approach used to identify and isolate cDNAs of differentially expressed genes in the field of cancer research (Yen, 2000; Su et al., 1997). These methodologies all have in common that one DNA population (driver) is hybridized in excess against a second population (tester) DNA to remove sequences present in both populations. In this project, we adapted the method for mRNA analysis to rapidly detect genes from an unknown virus. For this purpose, a subtraction hybridization-like method with a single-strand deletion technology was developed, and HCV was used as a hypothetical unrecognized virus in this study.

The traditional subtractive hybridization often requires large amounts of mRNA as starting material and it is commonly a challenging problem to obtain sufficient amounts of mRNA from the virus. In this project, high-quality full length

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cDNAs in large quantities were obtained by using a universal long-PCR based method (Clontech, BD, USA).

Normally, subtraction hybridization generated libraries typically contain some background clones representing non-differential genes (Liang, 2002). To overcome this problem a single-strand specific mung bean endonuclease in between the 1st and 2nd PCR was used which is ideally suited to rapidly reduce the number of false positives that could arise. More specifically it is single driver strands and linear amplified tester strands that are degraded. The double stranded products of the exponential amplification of the unique tester sequence remain unaffected. After reducing the background of the 1st PCR products, a nested PCR was performed by using the nested primers to further enrich the differentially produced sequences, which are from the tester but not from the driver.

After the subtracted cDNA library was obtained, it was cloned directly into a pGEM-T cloning vector, and plasmid DNAs from 96 random selected clones were analyzed. Differential reverse dot blot hybridization was carried out to determine whether the unique subtracted tester products represented the genomic region of the hypothetical unknown virus, in this case HCV. The hybridized clones were sequenced and sequences corresponding to the hypothetical unknown virus, HCV, were recovered. The inclusion of mung bean endonuclease treatment in the protocol precludes the need for multiple rounds of subtraction.

In recent years, methods for PCR amplification of long DNA fragments have advanced significantly. With intrinsic terminal transferase activity of a reverse transcriptase (RT) (Clontech, 2002; Petalidis et al., 2003) long PCR is capable of generating a full-length cDNA library from a small quantity of RNA. Additionally, the combined use of single-strand deletion technology in subtraction hybridization may eliminate or shorten steps required by traditional methods. This method will improve the ability to respond rapidly to any infectious disease outbreak and provide added value in the event of a bioterrorist event.

2. Material and methods

2.1. Experimental design, an overview

(1) The hypothetical unknown virus (HCV) was considered as an unknown mRNA in our study. (2) Unknown virus mRNA sequences present in the specimen were considered as tester (tester = HCV infected cell line). (3) The same type of specimen obtained from the same environment was considered as driver (driver = non-infected cell line). (4) Large quantities of cDNA were produced by universal long-PCR. (5) Our method is ideally suited to subtract out the most abundant common mRNA sequences present in both populations (tester and driver). (6) The method focused on a small number of unknown virus mRNA sequences present in the specimen and then enriched it by stoichiometry and kinetics. (7) A single-strand deletion technology limited the background from common sequences. (8) A subtraction cDNA library was constructed and displayed by differential reverse hybridization. (9) DNAs were sequenced for further characterization.

2.2. HCV infected cell lines

The human MOLT-4 acute lymphoblastic leukemia T cell line was obtained originally from the American Type Culture Collection (ATCC, Rockville, MD, USA; Lohmann et al., 1999; Wellnitz et al., 2002), and was maintained in RPMI 1640 ATCC modified medium (ATCC, Rockville, MD, USA) supplemented with 5% fetal bovine serum (ATCC, Rockville, MD, USA) in 75-cm^2 culture flasks at approximately 3×10^5 to 2×10^6 cells/ml. In vitro infection of the cell line was performed by using HCV RNA-positive serum from chronic HCV carriers. The cells were subcultured regularly with a split of 1:10 every week (Hu et al., 2003).

2.3. Total RNA preparation

Total cellular RNA was isolated from cell lines by a single-step extraction method with an acid guanidinium thiocyanate–phenol–chloroform mixture (Biotecx Laboratories Inc., Houston, TX, USA). The total RNA remained exclusively in the aqueous phase while proteins and DNA were extracted into an organic phase and interphase. The total RNA was precipitated from the aqueous phase by addition of an equal volume of 100% isopropanol, washed with 75% ethanol and solubilized in RNase free water. During RNA extraction, special care was taken to avoid cross-contamination by separating pre- and post-PCR work areas and using aerosol-proved tips. After extraction, a small aliquot of the RNA product was run on a gel to determine its quality.

2.4. mRNA preparation

Once the isolation of total RNA was complete, high quality mRNA was prepared from it with a Poly $(A)^+$ Tract mRNA Isolation System III (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, 500 μl of total RNA were heated at 65 °C for 10 min. 3 μl of biotinylated-oligo (dT) probe and 13 μl of 20× SSC were added and incubated at room temperature until completely cooled. The entire mixture was added to the tube containing the streptavidin paramagnetic particles and incubated at room temperature for 10 min. The mixture of mRNA-biotinylated oligo (dT) probe-streptavidin paramagnetic particles was captured on a magnetic stand and washed for four times with 0.1× SSC. mRNA was eluted from the streptavidin paramagnetic particles with 100 μl of RNase-free water, and then used as a template for cDNA synthesis.

2.5. Template-switching cDNA amplification

The template-switching activity of moloney murine leukemia virus (MMLV) reverse transcriptase with a mod-