DNaseI Treatment is a Prerequisite for the Amplification of cDNA from Episomal-Based Genes


The reverse transcriptase polymerase chain reaction (RT-PCR) (5) and the differential display of mRNA (6) are highly sensitive tools for the analysis of gene activity. Both methods rely on the reverse transcription of RNA followed by PCR-based amplification of cDNA. Even minor DNA contamination in the initial RNA preparation is a potential hazard that may lead to false-positive signals or competition effects. Commercial kits for the preparation of total RNA or mRNA give reliable results without apparent contamination with genomic DNA. However, here we report that these kits do not remove extrachromosomal DNA present in human cell lines which are either infected with Epstein-Barr virus (EBV) or are stably transfected with an EBV replicon-based plasmid, and we demonstrate that DNaseI treatment of the RNA is a pivotal step before cDNA generation.

EBV is associated with a number of human cancers and is able to immortalize primary human B-cells in vitro, leading to latently infected lymphoblastoid cell lines (LCLs). The viral genome persists in the cell as a 172-kbp extrachromosomal episome that is propagated by the EBV-encoded oriP/EBNA1 replicon. LCLs express a restricted number of viral genes including the EBNA-3, -4 and -6 proteins (EBNA 3 family), which are involved in the EBV-induced transformation of cells (reviewed in Reference 9). To analyze the impact of the EBNA 3 family on viral and human gene expression we used RT-PCR and differential display of mRNA from cells that express the EBNA-3, -4 and -6 proteins.

A 21.8-kbp shuttle vector was constructed by inserting the EBNA 3 family encoding the HindIII fragment of EBV strain M-ABA (8) into the HindIII cloning site of the mammalian expression vector EBO-pLPP. This vector takes advantage of the EBV-based replicon, enabling extrachromosomal replication of the episome (7). The HindIII shuttle vector was transfected into the human B-cell line dG75 using lipofectin according to procedures discussed in our recent report (11), and the bulk culture (EE346) was selected using hygromycin B. As shown by immunoblot, the EE346 cells stably express the EBNA-3, -4 and -6 proteins and the detailed results will be published elsewhere (N. Kienzle et al., unpublished).

Both total RNA and mRNA were prepared from EE346 cells and an LCL (K-Tutt cell line infected with EBV strain B95.8) using three commercially available RNA kits that were based on the guanidinium thiocyanate purification method (RNAGen® Total RNA Isolation System [Promega, Madison, WI, USA]; Total RNA Isolation Reagent [Advanced Biotechnologies, Surrey, England, UK]; and QuickPrep® Micro mRNA Purification Kit [Pharmacia Biotech, Uppsala Sweden]). One microgram of total RNA or 250 ng of mRNA were reverse-transcribed into first-strand cDNA (RT+) using oligo(dT) primers, deoxyribonucleoside triphosphate (dNTP) and SuperScript™ II enzyme according to the manufacturer’s guidelines (Life Technologies, Gaithersburg, MD, USA). For negative controls (RT−), the SuperScript II enzyme was omitted. The RT+/− samples were subjected to alkaline hydrolysis, purified with the QIAquick™ PCR Purification Kit (Qiagen, Chatsworth, CA, USA) and finally resuspended in 50 μL of water (1). Two-microliter aliquots were PCR-amplified in a 20-μL assay containing PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl2), 200 μM each dNTP, 0.5 μM each primer and 1 U of AmpliTaq® DNA polymerase (Perkin-Elmer, Norwalk, CT, USA). The ice-chilled samples were transferred to a preheated (85°C) GeneAmp™ PCR System 9600 instrument (Perkin-Elmer) and subjected to an initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 40 s and primer extension at 72°C for 40 s; a final extension was at 72°C for 5 min. The amplified samples were analyzed in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).
8.0) on a 3% agarose gel containing ethidium bromide.

Initially, the first-strand cDNA samples from EE346 and LCL were assayed for the presence of contaminating genomic DNA using the human β-hemoglobin-specific primer pair PC03 (5'-ACACAATTGTGTTCATC-TAGC) and PC04 (5'-CACTTATCATCAGTGTACC) (10). This primer pair spans a 119-bp region around the start codon of β-globin, which is only present at the genomic but not cDNA level. As indicated in Figure 1A, the 119-bp product could be amplified from 20 ng of genomic LCL DNA but not from the cDNA samples, indicating that there was no detectable chromosomal DNA contamination in either the mRNA or total RNA preparations.

Next, the samples were assayed for the presence of episomal DNA using the viral EBNA-6-specific primers RGIK1 (5'-GGAATGCAACCTCTGGGAA) and RGIK2 (5'-CCCGACCATCACAGTGCATT). These primers amplify a 146-bp fragment present in the second exon of EBNA-6 (position 99545 according to the EBV B95.8 sequence Reference 2). The RT+ as well as the RT- samples were analyzed to discriminate between cDNA and viral DNA sequences. As shown in Figure 1B, the EBNA-6 fragment was amplified in both the cDNA and RT- samples derived from total or messenger RNA, indicating the presence of contaminating episomal DNA in the RNA preparations from both EE346 cells and LCLs. Contamination of the reagents used by EBV sequences was ruled out because the cDNA derived from control cells transfected with EBO-pLPP contained no EBNA-6 DNA (data not shown). It has been reported that Tag DNA polymerase itself possesses some RT activity (4) that might have caused the EBNA-6 signal in the RT- samples shown in Figure 1B. However, the alkaline hydrolysis treatment in our purification protocol would have removed any possible target RNA.

In an attempt to eliminate the contaminating viral sequences, a DNasel treatment of the RNA preparation was introduced before reverse transcription. RNase-free DNase I (30 U; Boehringer Mannheim Australia, Castle Hill, NSW, Australia) was incubated with 40 µg of total RNA or 5 µg of mRNA in the presence of PCR buffer, 40 U of RNase-inhibitor (Boehringer Mannheim Australia) and 5 mM dithiothreitol for 50 min at 37°C in a total volume of 80 µL. The RNA was purified by a phenol/chloroform step, followed by isopropanol precipitation, and was used directly for RT-PCR.

As shown in Figure 1C, the EBNA-6 fragment could be PCR-amplified from the first strand cDNA only, but not from the RT- samples of EE346 cells and LCLs; therefore, the DNasel treatment was sufficient to remove contaminating episomal DNA. Two recent reports in this journal have pointed out the need for DNasel treatment of RNA before RT-PCR with chromosomal-encoded genes (3,12), but our method discriminates between chromosomal and episomal DNA contamination in both total RNA and mRNA populations. The data in this study emphasize the importance of the removal of episomal DNA from RNA preparations and is directly relevant to the study of viral gene expression.

REFERENCES


Use of a 96-Well Format for the Affinity Purification of Maltose-Binding Protein (MBP) Fusion Proteins


For the efficient expression of foreign sequences in Escherichia coli and subsequent purification of the resulting recombinant proteins, different sophisticated expression systems have been developed (8). For example, in the pMAL™.2 expression vectors, cloned sequences are inserted downstream of the E. coli malE gene, which encodes the maltose-binding protein (MBP), resulting in the production of an MBP fusion protein (5,7). Expression is under the control of the inducible “tac” promoter and the malE initiation signals (1,2). MBP fusion proteins can be purified efficiently using a single-step purification scheme that utilizes the affinity of MBP for its substrate (6). In principle, purification can be performed by affinity adsorption to amylose-resin beads. Although this approach is suitable for smaller numbers of clones, the handling of large numbers of various individual clones could be very laborious and time-consuming. Therefore, we developed a simplified method for the purification of recombinant proteins in a 96-well format.

The principle of our experimental approach was to use the polystyrene surface of the wells of microplates, instead of beads coated with a ligand for MBP, as a solid matrix for the affinity adsorption of MBP fusion proteins. In pilot experiments, we initially used maltose as the ligand for coating the wells. However, binding of MBP to wells that had been sensitized with maltose proved to be very inefficient,