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Urea-nuclease treatment of concentrated retrovirions preserves viral RNA and removes polymerase chain reaction-amplifiable cellular RNA and DNA

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Abstract

Cellular nucleic acids can interfere with the molecular cloning of retroviruses, a problem that is particularly serious with viruses propagated in lymphoblastoid cells that release large amounts of microvesicles and other cellular components. The approach taken to circumvent such problems involved first suspending viral pellets in water to allow any residual microvesicles to swell and perhaps lyse during overnight or longer incubation periods. Urea was then added to a concentration of 1.5–2.0 M to uncoil proteins that may protect nucleic acids from hydrolysis on the further addition of Micrococcal nuclease and ribonuclease A, both of which remain enzymatically active in molar urea solutions. The viral RNA was extracted and residual DNA removed by deoxyribonuclease I treatments. The utility of the method was demonstrated with two different retroviruses, a Moloney murine leukemia virus variant and Rous sarcoma virus, such that viral RNA thus purified was shown to be free of contamination by PCR-amplifiable cellular GAPDH mRNA and ribosomal RNA. This general approach should be applicable to viruses of any type in circumstances where contamination by cellular RNA and DNA poses a problem.

Keywords

Retroviruses; Ribonuclease; Urea; Micrococcal nuclease

1. Introduction

The molecular cloning of viral genomes, even from presumably purified virion preparations, can be confounded by contamination with cellular RNA and DNA. The problem particularly applies to retroviruses and especially those propagated in lymphocyte-derived cells, which release large amounts of vesicles and other cellular constituents that can persist even in purified virus preparations (Raposo et al., 1996; Bess et al., 1997; Gluschkof et al., 1997; Esser et al., 2001). An additional handicap in the RT-PCR cloning of retroviruses propagated in human cells is the predominant background of human endogenous retroviral sequences in cellular DNA, including 23,000–33,000 retropseudogenes (Gonçalves et al., 2000) (Löwer et al., 1996), some of which can be transcribed into RNA (Shih et al., 1989). It has been estimated that as much as 10% of the human genome may be composed of retroviral sequences (Temin, 1985). Another major source of contaminating cellular nucleic acids are the abundant ribosomal RNAs that are often difficult to hydrolyze with nucleases due to intimately

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associated ribosomal proteins that can protect the RNA. It is known that ribosomal RNA can interfere with the otherwise very effective method described by Löwer et al. (1993) of cloning the tRNA primer-binding site region at the 5' end of retroviruses. This interference problem has been a major impediment to the molecular cloning of the JHK virus, a small retrovirus produced in low yield in human B-lymphoblastoid cells (Grossberg et al., 1997).

A method to prepare concentrated retroviral samples is described herein that utilizes urea to lyse residual cellular vesicles and uncoil ribosomes and contaminating heterochromatin to permit the urea-resistant nucleases Micrococcal nuclease and RNase A to hydrolyze cellular nucleic acids external to the virus particles. The derivation of this method depended on finding a urea concentration that did not alter the integrity of the viral envelope and/or nucleocapsid that protects the inner viral RNA during nuclease treatment of cellular nucleic acids, allowing the subsequent extraction of intact viral RNA; any persisting cellular DNA was removed from the extracted RNA by DNase treatments. The utility of the method was demonstrated with two different retroviruses, Moloney murine leukemia virus (MMLV) and Rous sarcoma virus (RSV), and should be applicable to viruses of any type.

2. Materials and methods

2.1. Cells

The human B-lymphoblastoid cell line, designated DG-75 (UW), was originally obtained from Dr. Bill Sugden at the University of Wisconsin-Madison and grown in suspension culture as previously described; the DG-75 (UW) cells are chronically infected with the DG-75, a MMLV variant (Raisch et al., 1998). The human B-lymphoblastoid cell line JHK-3 was also used (Grossberg et al., 1997). Anchorage-dependant, secondary chicken embryo fibroblast (CEF) cultures (Rein and Rubin, 1968) were maintained in medium 199 (GIBCO Invitrogen, Carlsbad, CA) supplemented with 2% tryptose phosphate, 1% fetal bovine serum, 1% chick serum, and 1% antibiotic-antimycotic.

2.2. Viruses

The DG-75 virus, a xenotropic Moloney murine leukemia virus variant, is constitutively produced by DG-75 (UW) cell cultures (Raisch et al., 1998); its complete nucleotide sequence has been determined (GenBank accession no. AF221065) (Raisch et al., 2003). RSV infections were initiated in CEF cultures by transfection with plasmid pAPrC, an infectious proviral clone of the PrC strain of RSV (Meric and Spahr, 1986).

2.3. Urea-nuclease protocol

For RSV and DG-75 virus preparations, 160-200 ml crude cell culture supernatant from virus-infected cells was subjected to slow-speed centrifugation, passed through 0.45- and 0.22- μ m filters, and layered onto a 20% sucrose cushion, followed by centrifugation at $100,000 \times g$ for 1 h at 4 °C. The resulting viral pellet was re-suspended in 100 μ l of 10 mM Tris-HCl, pH 8.0, and usually stored 1 day or as long as 8 days at 4 °C. Urea was then added to a final concentration 1.5 M (Sigma-Aldrich, St. Louis, MO), followed by addition of Micrococcal nuclease (Amersham Pharmacia, Piscataway, NJ) and RNase A (Sigma-Aldrich, St. Louis, MO) to final concentrations of 4-8 U/ μ l and 0.5 U/ μ l respectively, and the samples were incubated at 37 °C for 60 min. After the addition of the RNase inhibitor Superase-In (20 units) (Ambion, Austin, TX), the viral RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, ethanol-precipitated, resuspended in 20 μ l of DEPC water, and treated with DNase I (0.1-1 U/ μ l) (Promega, Madison, WI) for 30 min at 37 °C. The RNA was then re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1), centrifuged through a MicroSpin S-200HR column, ethanol-precipitated, and the DNase I treatment process was

repeated two to three times. The final RNA was re-suspended in 20 μ l of DEPC-treated RNase-free water.

2.4. Reverse transcription and polymerase chain reaction

Viral RNA (3.5 μ l) was heated to 90 °C for 2 min, immediately cooled on ice, collected by brief centrifugation, and added to a 20- μ l reaction mixture containing 100 ng/ μ l hexamer primers (Gibco-BRL, Rockville, MD) and 200 U/ μ l MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) in accord with the manufacturer's recommended conditions. The mixture was incubated at 37 °C for 1 h, and the products were purified by phenol/chloroform/isoamyl alcohol extraction, passed through a MicroSpin S-200HR column, and ethanol-precipitated. For PCR, 3-5 μ l of either DG-75 cDNA or RSV cDNA was amplified in 50- μ l reaction mixtures containing 20 pmol primers and 5 U Taq DNA polymerase (Invitrogen) according to the manufacturer's conditions. The PCR program was as follows: 94 °C 30 s, 55 °C 30 s, 72 °C 1 min, 35 cycles, and extended at 72 °C for 10 min. The PCR primers used for DG-75 virus, RSV, ribosomal RNA, human and chicken GAPDH, and their product sizes are given in Table 1.

2.5. Product-enhanced reverse transcriptase (PERT) assay

The ultrasensitive PCR method of Pyra et al. (1994) with MS-2 bacteriophage RNA was followed as described and included positive and negative controls.

2.6. DNA sequencing and data analysis

PCR products were cloned in the pGEM-T vector (Promega, Madison, WI) and treated with the Big-dye Terminator kit (Applied Biosystems, Foster City, CA) for sequencing in an ABI DNA Sequencer. The nucleotide sequences and homology searches in GenBank were analyzed with BLAST (NCBI) and GCG (Wisconsin Version 10.2-UNIX) software.

3. Results

The methodology described herein was developed with the xenotropic DG-75 variant of Moloney murine leukemia retro-virus (Raisch et al., 1998). Attempts to purify virus by conventional sucrose-gradient ultracentrifugation and purification methods recommended for retroviruses (Hammar, 1994) failed to eliminate cellular RNA and DNA contamination as assessed by PCR. To find the optimal conditions to eliminate contaminating cellular RNA, DNA, and microvesicles, all preparative and treatment steps were varied, including the ratio of volume of water to viral pellet for osmotic lysis, the concentrations and combinations of nucleases, and the need for repeated penultimate DNase I treatments. Titration of urea over a wide range of molarity (1.0-5.0 M) showed that urea concentrations ≥ 2.5 M apparently disrupted the viral nucleocapsid as well as the viral envelope such that the liberated DG-75 viral RNA was then presumably hydrolyzed by the added nucleases, inasmuch as PCR-amplifiable viral RNA was no longer detectable (data not shown).

3.1. Urea-nuclease treatment of the DG-75 Moloney murine leukemia (MMLV) retrovirus

Fig. 1 illustrates the contamination by human lymphoblastoid cellular ribosomal and messenger RNA following conventional virus isolation without the urea-nuclease treatment. For this, DG-75 virus was obtained from 200 ml of DG-75 (UW) Blymphoblastoid cell culture supernatant fluid, which contains virus and large amounts of microvesicles and cellular debris, which are known to adversely affect viral RNA detection (Löwer et al., 1993; Grossberg et al., 1997; Bess et al., 1997; Esser et al., 2001). Virus particles were isolated by ultracentrifugation at 100,000 $\times g$, the nucleic acids extracted, and different RNA species detected by RT-PCR. In addition to viral RNA (Fig. 1, lane 4), cellular GAPDH and rRNA were also readily detected

(lanes 2 and 6). A methodology was therefore necessary to remove nucleic acid contaminants from the lymphoblastoid cell virion preparations.

To test the utility of the urea-nuclease procedure, DG-75 virus-containing human lymphoblastoid cell culture supernatant fluid was subjected to ultracentrifugation, processed by the urea-nuclease protocol, and PCR amplification performed as described above. The expected 258-bp DG-75 virus product was efficiently generated (Fig. 2, lane 2), whereas the PCR products derived from ribosomal RNA and GAPDH mRNA were eliminated (lanes 4 and 6); the lack of GAPDH signal was not due to a general failure of the reaction since GAPDH product was generated from DG-75 (UW) cellular genomic DNA (lane 7). The sequence of the PCR-amplified and cloned DG-75 *pol* fragment had 100% homology with that in GenBank (accession no. AF221065) (Raisch et al., 2003). Similar results were obtained following the urea-nuclease treatment of DG-75 virus to which the supernatant fluid was added from B-lymphoblastoid JHK-3 cells, which produce unusually large amounts of microvesicles and cellular debris. Thus, the urea-nuclease treatment effectively eliminated PCR-amplifiable contaminating cellular RNA from the DG-75 viral pellet.

3.2. Urea-nuclease treatment of Rous sarcoma virus

Fig. 3 illustrates the contamination by cellular ribosomal and messenger RNA after conventional virus isolation of an unrelated retrovirus of avian origin, RSV, that was grown in anchorage-dependent chicken embryo fibroblasts (CEF). Following transfection of CEF with a full-length proviral clone (pAPrC), RSV reverse transcriptase (RT) activity increased daily in crude cell culture supernatant fluid to a maximum at 5 days (data not shown). Virus particles from day-5 harvests of supernatant fluids were then concentrated by ultracentrifugation and either processed or not through the urea-nuclease protocol. The RSV RNA extracted from virions that had not been exposed to the urea protocol was treated twice with DNase I to eliminate possible DNA contamination. RSV RNA was reverse-transcribed with hexamer primers and PCR-amplified with a primer set targeting the RSV *pol* gene. As shown in Fig. 3, a specific, appropriately sized PCR product was obtained with the RSV *pol* primers (lane 3), but contamination with rRNA (lane 5) and GAPDH mRNA (lane 7) was apparent. Genomic DNA contamination was absent (compare the minus-RT control lanes 2, 4, and 6 with lane 8); the control GAPDH cellular genomic DNA product (lane 8) is larger because the chicken gene contains two introns. Thus, conventional RSV virus isolation from CEF results in rRNA and mRNA contamination.

As shown in Fig. 4, the RSV preparation treated by the ureanuclease protocol was free of contaminating cellular rRNA (lane 5) or GAPDH mRNA and DNA (lanes 6 and 7) while positive for RSV *pol* (lane 3). RSV-specific PCR products were also obtained with *gag* and 5'-end (R region) primer pairs (data not shown). To verify their specificity, the RSV PCR products from the three regions of the RSV genome (*pol*, *gag*, and the 5' R region) were cloned and sequenced, and their homology with the RSV sequences in the GenBank (accession no. AF033808) was shown to be 100% (data not shown). These data demonstrate the utility of the urea-nuclease procedure to recover PCR-amplifiable, RSV gene-specific products free of contaminating cellular RNA and DNA as assessed by a lack of detectable rRNA, GAPDH mRNA, and DNA.

4. Discussion

Virus preparations are often contaminated by cellular components such as microvesicles, organelles, proteins, and nucleic acids, and this is especially true for retroviruses, e.g., the human immunodeficiency viruses (HIV), or human T lymphocyte viruses (HTLV), grown in lymphocyte-derived cells, which release large amounts of vesicles and other cellular constituents (Raposo et al., 1996; Bess et al., 1997; Esser et al., 2001). Because of the

ultrasensitivity of PCR, the presence of cellular nucleic acids can interfere with the molecular cloning of new viruses, especially those produced in low yield. The methodology described herein was developed to address problems encountered with the JHK retrovirus that is propagated in B-lymphoblastoid cells (Grossberg et al., 1997). The approach taken may well be applicable to viruses of any nature.

Like the histones in nucleosomes (Kornberg, 1977), ribosomal proteins are intimately associated with rRNA such that nucleases may not be able to gain access to degrade all the associated RNA; rRNA constitutes 80% of cellular RNA and is a problematic source of contamination. Jackson and Chalkley (1975) found that treatment with 4-5 M urea could unfold nucleosomes, by virtue of urea's hydrogen-bond-rupturing effect on tertiary protein structure, to which Micrococcal (Staphylococcal) nuclease was added to efficiently hydrolyze the DNA that was otherwise protected by protein. Ribonuclease A and Micrococcal nuclease maintain their activity in the presence of molar urea. It was reasoned that a similar approach might be taken to deal with the contamination of concentrated retroviral preparations by nuclease-resistant rRNA and cellular DNA.

The urea-nuclease procedure can be easily completed in less than 2 days. The critical steps in the protocol are: (1) slow-speed ($1000 \times g$) centrifugation and membrane filtration to remove cells from virus-infected cell culture supernatant fluids (especially from lymphoblastoid cells grown in suspension); (2) ultracentrifugation through a 20% sucrose cushion to separate virions of higher density from the membranes, nucleic acids, and microvesicles of lower density; (3) resuspension of the viral pellet in water for at least 16 h to promote osmotic swelling and lysis of contaminating microvesicles (unlike concentrated retrovirions which maintained integrity in water for as long as 8 days); (4) addition of urea to a final concentration of 1.5-2.0 M in order to unfold ribosomal proteins and heterochromatin that can act to protect nucleic acids from enzymatic hydrolysis by nucleases (the exact urea concentration may need to be determined for any given virus); (5) utilization of nucleases known to be enzymatically active in molar urea solutions, specifically Micrococcal nuclease and RNase A; (6) enzymatic digestion of the residual DNA that contaminates the extracted RNA, a procedure which must be done at least twice inasmuch as one treatment was not adequate for reliably removing DNA, sometimes requiring four DNase treatments depending on the preparation.

It is not known whether retroviral envelope and nucleocapsid are differentially susceptible to dissolution by urea. Urea concentrations greater than 2.5 M (with added RNase and Micrococcal nuclease) must have destroyed the integrity of both membranes inasmuch as no viral RNA could then be detected by PCR. If the viral envelope were not able to withstand the urea treatment, the viral nucleocapsid must be sufficiently resistant to 1.0-2.0 M urea treatment to protect the viral RNA from the added nucleases since virus-specific sequences could then be PCR-amplified and cloned. A method to prevent RT-PCR amplification of contaminating DNA sequences by treatment of cDNA first strands with DNase I (Flohr et al., 2003) provides another approach to deal with DNA contamination. The urea-nuclease procedure should be applicable to preparations of viruses of any type where contamination with cellular RNA and DNA poses a problem.

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Fig. 1.

Cellular nucleic acid contamination of a Moloney murine leukemia virus (strain DG-75) preparation upon conventional virus isolation. Nucleic acids were extracted from a $100,000 \times g$ DG-75 viral pellet that was concentrated from the supernatant fluid of human B-lymphoblastoid cell cultures, and RT-PCR amplification was performed by universal hexamer RT priming (no RT controls in lanes 1, 3, and 5) and PCR with primers to GAPDH (lanes 1, 2, 7, and 8), DG-75 virus (lanes 3 and 4), and rRNA (lanes 5 and 6). A sample of DG-75 cellular genomic DNA as an additional control was amplified in lane 7, and water was substituted for DNA in lane 8. Note the presence of contaminating cellular GAPDH and ribosomal RNA in the virus samples. M, 100 bp standard markers.

Fig. 2.

The urea-nuclease procedure effectively eliminates cellular RNA contamination of DG-75 virus prepared from human B-lymphoblastoid cell cultures. A $100,000 \times g$ pellet of DG-75 virus was subjected to the 1.5 M urea-nuclease protocol, and RT-PCR amplification was performed by universal hexamer RT priming (no RT controls in lanes 1, 3, and 5) and PCR with primers to DG-75 (lanes 1 and 2), rRNA (lanes 3 and 4), and GAPDH (lanes 5-8). A sample of DG-75 cellular genomic DNA was amplified in lane 7, and water was substituted for DNA in lane 8. Note that the lane order is different from Fig. 1. No cellular RNA contamination of DG-75 virus is observed. M, 100 bp standard markers. The positions of the 200 and 300 bp markers are shown at left.

Fig. 3.

Cellular RNA contamination of conventional avian Rous sarcoma virus preparations. Nucleic acids were extracted from the $100,000 \times g$ RSV viral pellet concentrated from the supernatant fluid of transfected chicken embryo fibroblasts (CEF), and RT-PCR amplification performed by universal hexamer priming (no RT controls in lanes 2, 4, and 6) and PCR with primers to RSV *pol* (lanes 2 and 3), ribosomal RNA (lanes 4 and 5) and GAPDH (lanes 6-9). A sample of CEF DNA was amplified in lane 8, and water substituted for DNA in lane 9. The chicken GAPDH sequence flanked by the primers contains two introns, hence the larger size for the genomic product. Note the contaminating cellular rRNA and GAPDH mRNA in the virus samples. M, 100 bp standard markers.

Fig. 4.

The urea-nuclease procedure effectively eliminates cellular RNA contamination of RSV prepared from avian cells. RSV particles were isolated from transfected chicken embryo fibroblasts (CEF) by ultracentrifugation and subjected to the 1.5 M urea-nuclease protocol. RT-PCR amplification was performed by universal hexamer RT priming (no RT controls in lanes 2, 4, and 6) and PCR with primers to the RSV *pol* gene (lanes 2 and 3), rRNA (lanes 4 and 5), GAPDH (lanes 6-9); a sample of CEF genomic DNA was amplified in lane 8, and water was substituted for DNA in lane 9. No cellular RNA contamination of RSV is observed. M, 100 bp standard markers.

Table 1

PCR primer pairs and product sizes

Name	Target	Sequence (5' to 3')	Product size (bp)
GAPDH-3	Human GAPDH	TGATGACATCAAGAAGGTGGTGAAG	240
GAPDH-4		TCCTTGGAGGCCATGTGGGCCAT	
GAPDH(ch)-f	Chicken GAPDH ^a	TGATGATATCAAGAGGGTAGTGAA	240, 793
GAPDH(ch)-r		TCCTTGGATGCCATGTGGACCAT	
rRNA-f	18S rRNA ^b	TCTGCCCTATCAACTTTC	221
rRNA-r		AGTGGACTCATTCCAATTAC	
DG-75-f	DG-75 virus <i>pol</i> ^c	AATGCTCCTAGACACTGACC	258
DG-75-r		CAGATTACCTCGGTCTCAG	
RSV-f	RSV <i>pol</i> ^d	ACCGCTTACTGCATGATTTG	440
RSV-r		AGGCGAAATAGTGAACCCG	

^aThe GAPDH chicken gene has two introns such that the genomic DNA PCR product is larger than that of the mRNA.

^bThe primers for the human and chicken ribosomal RNA sequences are identical.

^cThe DG-75 virus *pol* primers amplify nucleotides 3941-4179.

^dThe RSV *pol* primers amplify nucleotides 2705-3144.