A simple and rapid reverse transcriptase assay for the detection of retroviruses in cell cultures

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Abstract

Reverse transcriptase (RT) is a good diagnostic tool for the detection of retroviruses. We have developed a simple and rapid assay for RT activity in culture supernatants. A 370-base RNA sequence from the tetracycline-resistance gene in pBR322 plasmid DNA was used as a template for RT-mediated cDNA synthesis. To detect the resultant cDNA, we used the nested polymerase chain reaction. A sensitivity test using purified recombinant RT of human immunodeficiency virus type 1 demonstrated that the detection limit of this method was 10^{-7} – 10^{-8} units of RT activity in 20 μ l of a test sample (2×10^{-9} – 2×10^{-10} units ml^{-1}). This method detected RT activity in unconcentrated supernatants of cell cultures infected with human T-cell leukemia virus, Moloney murine leukemia virus, Moloney murine sarcoma virus, or Rous sarcoma virus. This nonisotopic method provides results within 10 h and is useful for quality control to detect retroviruses in cell cultures.

Abbreviations: HIV – human immunodeficiency virus, HTLV – human T-cell leukemia virus, PCR – polymerase chain reaction, RT – reverse transcriptase

Introduction

Retroviruses are causative agents of immunodeficiency, anemia, leukemia, lymphoma, and sarcomas in humans and animals (Schupbach, 1989). In recent years, awareness has grown of the biohazards of retrovirus-infected cell cultures (Harbour and Woodhouse, 1990; Losikoff et al., 1991; Shadle et al., 1995) and of the need to exclude such potential biohazards from cell cultures by performing tests for infectious retroviruses such as human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 (HTLV-I). Furthermore, some retroviruses remain undetected in cell cultures as latent contaminants and can cause serious complications in the interpretation of experimental results. Therefore, tests for the entire retrovirus family are necessary for quality control of cell cultures (Hane and Drexler, 1993; Hay et al., 1996). Retroviruses possess a characteristic enzyme,

reverse transcriptase (RT), which has two major enzymatic activities: A DNA polymerase activity, which uses either RNA or DNA as a template; and a nuclease activity, termed ribonuclease H, which degrades the genomic RNA in RNA-DNA hybrid form (Baltimore, 1970; Temin and Mizutani, 1970; Katz and Skalka, 1994). RT is encoded by retrovirus RNA and is essential for retrovirus replication. Assays for RT activity, therefore, have been used as a tool for the detection of retroviruses.

Conventional assays for RT activity are as follows: Virus particles in test samples are concentrated by ultracentrifugation or polyethylene glycol precipitation and solubilized by a detergent, then RT activity is detected by incorporation of [³H] or [³²P]-labeled deoxythymidine triphosphate into DNA in the presence of a synthetic homopolymer RNA template such as poly[A] (Mizutani et al., 1974; Inder et al., 1974; Lee et al., 1987). The conventional assays, however, are not easy to perform because they involve the

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(A)

(1) Preparation of RNA template

(2) RT assay

(B)

Figure 1. (A) An outline of the preparation of RNA template and the RT assay. To prepare a 370-b RNA template, a 370-bp DNA fragment inside the tetracycline-resistance gene of pBR322 plasmid was amplified by PCR using the primer pair of T7-F1 and R1. The amplified 370-bp DNA fragment was transcribed into the RNA template for RT reaction by T7 RNA polymerase. RT reaction was performed using the 370-b RNA template. After RT reaction, synthesized cDNA was amplified by nested PCR and detected by agarose gel electrophoresis. Primer R1 was used for both RT reaction and the first-step PCR. (B) The constituents for RT reaction and the first-step PCR in a single tube. Virus particles were disrupted by Nonidet P-40, then RT reaction occurred in the upper phase. After heating the tube at 95 °C for 3 min, the resultant cDNA was mixed with the first-step PCR buffer in the lower phase.

use of isotopes, special equipment, and troublesome procedures. In recent years, nonisotopic assays have been developed using biotin-avidin or digoxigenin for labeling nucleotide triphosphates and enzyme-linked immunosorbent assay or polymerase chain reaction (PCR) for detecting the resultant cDNA (Cook et al., 1992; Eberle and Seibl, 1992; Silver et al., 1993; Suzuki et al., 1993; Pyra et al., 1994; Heneine et al., 1995; Hughes et al., 1995). Although most of these methods allow comparatively simple and safe detection of RT activity, they still involve steps such as a procedure for virus concentration (Somogyi et al., 1990). Here we have developed a simple and rapid RT assay, in which an *in vitro*-transcribed heteropolymeric RNA sequence is used as a template and the resultant cDNA is detected by nested PCR. This assay should be useful for the detection of retroviruses in cell cultures.

Materials and methods

An outline of preparation of RNA template and RT assay is shown in Figure 1A.

Preparation of the RNA template

A 370-base (b) RNA corresponding to a DNA sequence that includes the area between the *Bam*HI 375 site and *Sal*I 651 site of the pBR322 tetracycline-resistance gene was used as a template for RT. First, we amplified 370-base pair (bp) DNA from pBR322 plasmid by PCR using the primer pair of T7-F1 (5'-GGTACCTAATACGACTCACTATCAC-TATCGACT-ACGCGAT) and R1 (5'-AGTCATGCCCCGCGC-3'). T7 promoter sequence (22 b; 5'-GGTACCTAATACGACTCACTAT-3') was added to the end of the 5' forward primer (F1) to allow *in vitro* transcription of the PCR product. The PCR was carried out with 100 μ l of a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM deoxynucleoside triphosphate (each), 30 pmol of each primer (T7-F1 and R1), and 2.5 units of Taq polymerase (Takara Shuzo). The reaction was cycled 30 times with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min. Next, the PCR product was transcribed by T7 RNA polymerase reaction using RiboMAX™ Large Scale RNA Production Systems (Promega) according to the manufacturer's instructions. The DNA template in the RNA preparation was digested three times with 1 unit of DNase I (Promega) against 1 μ g of template DNA at 37 °C for 30 min, then DNase I was

inactivated by heating at 95 °C for 10 min. The RNA preparation was extracted twice with TE buffer (pH 8.0)-saturated mixture of phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v) and precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 99.5% ethanol. The pellet was washed twice with 80% ethanol, air-dried, then resuspended in diethylpyrocarbonate-treated H₂O.

RT assay

RT reaction and the first-step PCR were performed in a single 0.5-ml PCR tube that contained a wax bead for PCR (HotStart 100, Molecular Bio-Products) as shown in Figure 1B. The first-step PCR buffer (50 μ l) consisting of 5 mM Tris-HCl (pH 8.3), 25 mM KCl, 1.5 mM MgCl₂, 30 pmol of 5' forward primer (F1: 5'-CACTATCGACTACGCGAT-3') and 2.5 units of Taq polymerase was added to the tube. The tube was kept at 70 °C for 3 min to melt the wax bead, then at 4 °C for 3 min to allow the wax layer to harden over the lower layer. An RT reaction mixture (40 μ l) consisting of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.07% (v/v) Nonidet P-40, 1 mM EGTA, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate (each), 30 pmol of reverse primer (R1), 10 units of RNase inhibitor (Wako Pure Chemicals), and 10 ng of the RNA template was poured on the wax. Cell culture supernatants (20 μ l) to be tested were added to the RT reaction mixture. To examine the sensitivity of the RT assay, 10⁻³–10⁻⁸ units of purified recombinant RT of HIV-1 (Dupont) in 20 μ l of RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum was used. One unit of HIV-1 RT was defined as the amount of enzyme incorporating 1 nmol of ³H-dTMP into acid products in 10 min at 37 °C. The RT reaction was performed with a DNA thermal cycler (Perkin Elmer). The parameters were as follows: RT reaction at 37 °C for 180 min, denaturation of RT and melting of the wax at 95 °C for 3 min, cooling at 0 °C for 3 min.

After the RT reaction, nested PCR was performed to detect the resultant cDNA. The first-step PCR was cycled 35 times with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min. The first-step PCR product (2 μ l) was mixed with 98 μ l of the second-step PCR buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM deoxynucleoside triphosphate (each), 30 pmol of each internal primer (F2: 5'-GCCTATATCGCCGACATC-

3', R2: 5'-CTTATGCGACTCCTGC-3') and 2.5 units of Taq polymerase. The second-step PCR was carried out with the same cycling as the first-step PCR. The second-step PCR product (10 μ l) was electrophoresed in 2.0% agarose gels and stained with ethidium bromide. Only samples with a clearly visible band of the estimated size, namely, about 200 bp, were considered to be positive.

Colorimetric RT assays

For comparison of sensitivity, two commercially available kits for RT assay were used. One is an enzyme-linked oligonucleotide sorbent assay (ELOSA) RT assay (RT-Detect™ NEN™ Life Science Products) and the other is a non-radioactive (non-RI) RT assay (Boehringer Mannheim). In the ELOSA RT assay (Greene et al., 1992), two different oligonucleotide probes specific for cDNA, a biotinylated capture probe and a horseradish peroxidase-conjugated reporter probe, were used to detect the cDNA synthesized by RT. The hybrids of cDNA and probes were trapped on streptavidin-coated micro-well modules. In the non-RI RT assay (Eberle and Knopf, 1996), the nonisotopic label digoxigenin and biotin are incorporated into cDNA via polymerization by RT. The cDNA trapped on streptavidin-coated micro-well modules was labelled by horseradish peroxidase-conjugated antibody against digoxigenin. In the two RT assays, the cDNA were quantified with an enzyme-linked immunosorbent assay. All the procedures were performed according to the manufacturers' instructions.

Cell culture

Fourteen cell lines used are specified in Table 1. NIH3T3-Mo-MuLV and TB-Mo-MuSV were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% (v/v) fetal calf serum. The other 12 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The culture supernatants were taken 3 to 6 days after the last subcultivation and kept at -80 °C until the assay.

Results and discussion

Sensitivity and specificity of this method

To determine the sensitivity of the RT assay, purified recombinant RT of HIV-1 having 10 units ml⁻¹ of

Figure 2. Sensitivity of the RT assay. Purified recombinant RT of HIV-1 was serially diluted in RPMI 1640 medium supplemented with 10% fetal calf serum. The test samples (20 μ l) indicated in the figure were assayed. Molecular sizes (100-bp ladder) are indicated on the left-hand side.

RT activity was used. A series of enzyme solutions diluted 10-fold with RPMI-1640 containing 10% fetal calf serum was subjected to the RT assay. A single band at about 200 bp was apparent from the samples at 10^{-3} – 10^{-7} units in 20 μ l of test sample, indicating that the detection limit of this assay was 10^{-7} – 10^{-8} units (2×10^{-9} – 2×10^{-10} units ml⁻¹) of HIV-1 RT activity (Figure 2). The sensitivity of the RT assay was compared with two colorimetric RT assays using the commercially available kits, the ELOSA RT assay and the non-RI RT assay. The detection limit for HIV-1 RT activity of the two assays was 100 to 1000-fold lower (i.e., 2×10^{-7} units ml⁻¹) than that of our RT assay.

To examine whether the method is applicable to the detection of RT activity released from cells infected with retroviruses, supernatants of human T-cell lines, MT-1 and MT-2, were examined. MT-1 and its subclone MT-2 are known to carry HTLV-I (Hinuma et al., 1981; Yoshida et al., 1982). We confirmed the presence of HTLV-I in the cell cultures by immunocytochemistry using a mixture of monoclonal antibodies to p19, p28 and GAG of HTLV-I (diluted 1:20, Chemicon). Most MT-2 cells (>95%) expressed

Table 1. Survey of RT activity in culture supernatants

Cell line	Registration No.	Species	Origin	Result
MT-1		human	acute lymphoblastic leukemia	+
MT-2		human	acute lymphoblastic leukemia	+
XC	IFO 50017	rat	sarcoma	+
NIH-Mo-MuLV		mouse	fibroblast	+
TB-Mo-MuSV		mouse	fibroblast	+
CCRF-HSB2	JCRB 0031	human	acute lymphoblastic leukemia	-
BALL-1	JCRB 0071	human	acute lymphoblastic leukemia	-
TALL-1	JCRB 0086	human	acute lymphoblastic leukemia	-
A3/KAW	JCRB 0101	human	malignant lymphoma	-
PEER	JCRB 0830	human	acute lymphoblastic leukemia	-
MOLT-3	JCRB 9048	human	acute lymphoblastic leukemia	-
MEG-O1	IFO 50151	human	megakaryoblastic leukemia	-
MOLT-4	IFO 50362	human	acute lymphoblastic leukemia	-
CCRF-CEM	IFO 50412	human	acute lymphoblastic leukemia	-

+ = Detected; - = not detected.

IFOxxxxx and JCRBxxxx indicate the registration numbers of the cell lines at IFO Cell Bank and Health Science Research Resources Bank, respectively.

the HTLV-I antigens whereas only 2% of MT-1 cells expressed them. Two lines of human T-cells, MOLT-4 and CCRF-CEM, were negative for the HTLV-I antigens. The results of RT assay on the four cell lines were consistent with those of immunocytochemistry: MT-1 and MT-2 were positive, and MOLT-4 and CCRF-CEM were negative (Figure 3). The results indicate that this RT assay was a specific diagnosis for RT activity. The retroviruses might be partially disrupted by the nonionic detergent (Nonidet P-40) in the RT reaction mixture, so that RT released from virus particles could be detected. The lentivirus subfamily of retroviruses such as HIV-1 might be readily detected, but oncoviruses such as HTLV-I are much more difficult to detect presumably because of their low RT activity and cell-associated nature (Lee et al., 1987; Cook et al., 1992; Eberle and Seibl, 1992; Suzuki et al., 1993). This method could detect RT activity in the culture supernatants of MT-1 cells, indicating its high sensitivity.

Detection of RT activity derived from different retroviruses by this method

To confirm that this method can be applied to other retroviruses besides human retroviruses, we examined supernatants of three cell lines that were infected with murine or avian retroviruses. NIH-Mo-MuLV was derived from a mouse cell line, NIH 3T3, infected with Moloney murine leukemia virus (Ball et

al., 1973). TB-Mo-MuSV was derived from a mouse cell line, TB, infected with Moloney murine sarcoma virus (Ball et al., 1973). XC was derived from a transplantable tumor induced in newborn rats by the injection of avian Rous sarcoma virus (Svoboda, 1960). The method could detect RT activity in the supernatants of these cell cultures. The RT of mammalian C-type retroviruses, such as Moloney murine leukemia virus, shows a preference for Mn^{2+} rather than Mg^{2+} while employing homopolymer templates (Zhan and Crouch, 1997). The addition of 2, 5, or 10 mM Mn^{2+} to the RT reaction mixture did not affect the sensitivity of the method for detecting purified recombinant RT of Moloney murine leukemia virus (Boehringer Mannheim). The results indicate that this method was applicable to the detection of different taxonomic classes of retroviruses.

Applicability of the RT assay to the quality control of cell cultures

Many retroviruses are endogenous, being present in source materials used for initiation of cell cultures, but some are extraneous and are accidentally introduced into cell cultures through the media used. Thus, all cell cultures should be tested for the presence of retroviruses. To make RT assay more convenient and reduce the chance of carry-over contamination, we set up the RT reaction and the first-step PCR in separate compartments of a single tube. Furthermore, we

Figure 3. Detection of RT activity in culture supernatants. The culture supernatants (20 μ l) of MT-1, MT-2, CCRF-CEM, or MOLT-4 were examined. Molecular sizes (100-bp ladder) are indicated on the left-hand side.

employed nested PCR method instead of Southern blotting (Heneine et al., 1995) to detect the first PCR products. The RT assay could be completed within 10 h. For quality control of cell lines preserved in the IFO cell bank and the Health Science Research Resources bank, we examined nine cell lines derived from human leukemia/lymphomas and found them to be negative (Table 1).

This assay indicates whether RT activity is present in culture supernatants, but not whether retrovirus particles being released. To detect retrovirus production by cell cultures, RT activity in the virus-particle fraction separated on a sucrose density gradient should be examined. The RT assay is suited to the processing of numerous samples and useful for the first screening of retroviruses in cell cultures.

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