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A Simple, General Method for Detecting Retroviral RNAs Expressed in Cells

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A simple, efficient method has been developed for detecting retroviral RNAs expressed in cells. In this method, total RNAs or poly A^+ RNAs extracted from various human cells are separated by electrophoresis and hybridized with synthetic oligonucleotides corresponding to the 3′-terminal 18 nucleotides of various tRNAs. Genomic and subgenomic RNAs of HTLV-I and HTLV-II in virus-infected cells and of xenotropic murine leukemia virus expressed in human lung cancer cells were easily detected with the $tRNA^{Pro}$-derived oligonucleotide probe. This technique can be used to search for unidentified retroviruses expressed in human cancer cells and tissues.

Key words: Retrovirus — Primer binding site — Primer tRNA — Viral RNA — Xenotropic murine leukemia virus

Genomic retroviral RNA contains an 18-nucleotide sequence (primer binding site, PBS) that is complementary to the 3′-end of a specific host tRNA (primer tRNA), on the immediate 3′-side of the U5 sequence.[1] The primer tRNA binds to the PBS and is used as a primer for initiation of genomic RNA-dependent DNA synthesis by reverse transcriptase.[1] Retroviruses use various tRNAs as primers; most avian retroviruses use $tRNA^{Trp}$, whereas mammalian type C retroviruses including HTLV-I and -II use $tRNA^{Pro}$ and mouse mammary tumor virus uses $tRNA^{Lys}_{UUU}$.[2] Recently, several endogenous retrovirus-like sequences which have PBS sequences complementary to $tRNA^{Glu}$,[3] $tRNA^{Arg}$,[4] $tRNA^{His}$,[5] $tRNA^{Ile}$,[6] or $tRNA^{Lys}_{CUU}$[7] have been isolated from the human genome.

We and others recently reported a new method for isolating genomic clones of endogenous retrovirus-like sequences from a human genomic library using tRNA as a hybridization probe.[8,9] We obtained three kinds of human endogenous retrovirus-like sequences by this method.[8] In this study, we applied this method for detecting retroviral RNAs expressed in various human cells.

MATERIALS AND METHODS

Cell lines Human cell lines were obtained from the following sources: T-cell leukemia cell lines (Jurkat and H9), B-cell lines (Raji and Wil 2), HTLV-I-infected cell lines (MT-2 and HUT102), HTLV-II-infected cell lines (Ton 1 and Wil 2-43) and HeLa cells from Drs. K. Shimotohno and H. Tanaka of the National Cancer Center Research Institute, Tokyo; a lung giant-cell carcinoma cell line (Lu-65) from Dr. S. Hirohashi of the National Cancer Center Research Institute, Tokyo; a colon adenocarcinoma cell line (SW-480), a lung large cell carcinoma cell line (PC-13) and a urinary bladder carcinoma cell line (T-24) from Dr. T. Okumoto of the Research Laboratories, Yoshitomi Pharmaceutical Industries, Ltd., Saitama; an osteosarcoma cell line (OST) and acetabular fibrosarcoma cell line (HT-1080) from Dr. K. Tomita, Kanazawa University, Kanazawa; an oral epidermoid carcinoma cell line (KB) from Dr. Y. Yanagi, Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., Osaka; T-cell acute lymphoblastic leukemia cell lines (CCRF-CEM, MOLT-3, MOLT-4 and TALL-1), B-cell acute lymphoblastic leukemia cell lines (KG-1, CCRF-SB and BALL-1), a histiocytic lymphoma cell line (U-937), chronic myelogenous leukemia cell lines (K-562 and KU812), acute monocytic leukemia cell lines (J-111 and THP-1), a promyelocytic leukemia cell line (HL60RG), an erythroleukemia cell line (HEL), a B lymphoblast cell line (IM-9), a myeloma cell line (RPMI-8226), peripheral lymphocyte cell lines (HLCL-1 and RPMI-1788) and a leukemia cell line (KY821) from the Japanese Cancer Research Resources Bank.

Hybridization probes The 17 octadecanucleotides listed in Table I were synthesized in a DNA synthesizer (Applied Biosystems 380A). Oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.[10] The recombinant plasmid pLCAT-L containing the 5′-LTR of HTLV-I was kindly provided by Dr. M. Seiki of Kanazawa University, Kanazawa. The purified DNA

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fragment was labeled with ^{32}P with an Amersham multiprime DNA labeling system.

RNA preparation and Northern blotting Total RNA was extracted from cells by the guanidinium/CsCl method.[10] Poly A^+ RNA was selected by oligo (dT)-cellulose column chromatography.[10] Total RNA (10 μg) or poly A^+ RNA (2.5 μg) was denatured and separated in 1.2% agarose gel containing formaldehyde. The RNA was transferred from the gel to a nitrocellulose membrane and hybridized with an oligonucleotide probe. Hybridization was carried out in 6×SSC (1×SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate), 5×Denhardt's solution,[11] 0.1 mg/ml of salmon sperm DNA, 10% dextran sulfate and 0.1% SDS for 18 h at 42°C. The hybridized filter was washed with two changes of 2×SSC containing 0.1% SDS for 15 min each time at 42°C and then with 0.1× SSC containing 0.1% SDS at 42°C for one hour. Filters were antoradiographed with Kodak XAR films for one day (Fig. 1C), 2 days (Figs. 1B, 2A, 2B and 4A), 3 days (Fig. 4B) or 4 days (Fig. 1A) at −70°C with intensifying screens.

cDNA cloning and DNA sequencing A cDNA library of Lu-65 cells was prepared from 5 μg of poly A^+ RNA with oligo dT primer using a cDNA synthesis system plus (Amersham) and cDNA cloning system-λgt10 (Amersham). Recombinant phages were screened with the $tRNA^{Pro}$-derived oligonucleotide probe. The hybridization and washing conditions were the same as for Northern blotting. *Eco*RI fragments from purified phage clones were subcloned in pKH47 vectors.[12] The inserts were purified, digested with various restriction enzymes and subcloned into M13 mp10/11.[13] DNA sequence analysis was performed by the dideoxy-sequencing method of Sanger *et al.*[14] in an Applied Biosystems 370A DNA sequencer. All sequences shown were determined in both DNA strands.

RESULTS

Oligonucleotide probes for detecting retroviral RNAs The 3′-terminal 18-nucleotide sequence of primer tRNA is complementary to the primer binding site of retroviral genomic RNA.[1] We prepared 17 different 3′-terminal

Table I. List of Octadecanucleotides Used as Hybridization Probes

tRNA	Sequence	(Reference)
Pro	ATCCCGGACGAGCCCCCA	(24)
Trp	ATCACGTCGGGGTCACCA	(24)
Lys (CUU)	GCCCCACGTTGGGCGCCA	(24)
Lys (UUU)	GTCCCTGTTCGGGCGCCA	(24)
Glu	TTCCCGGTCAGGGAACCA	(24)
Arg	CTCCTGGCTGGCTCGCCA	(24)
His	ATCCGAGTCACGGCACCA	(24)
Ile	TCCCCGTACGGGCCACCA	(24)
Phe	TCCCGGGTTTCGGCACCA	(24)
Asp	TTCCCCGACGGGGAGCCA	(24)
Gln	ATCTCGGTGGGACCTCCA	(24, 25)
Thr	ATCTCGCTGGGGCCTCCA	(26)
Gly	TTCCCGGCCAATGCACCA	(24)
fMet	AACCATCCTCTGCTACCA	(24)
Tyr	TTCCGGCTCGAAGGACCA	(24)
Val	AACCGGGCGGAAACACCA	(24)
Met	TCCTCACACGGGGCACCA	(24)

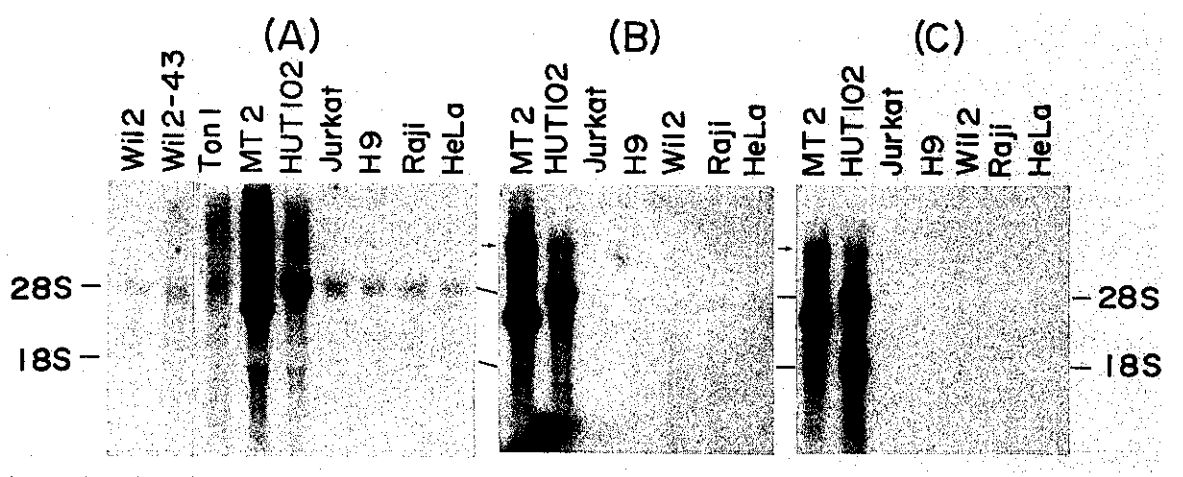

Fig. 1. Northern hybridization analysis of RNAs prepared from various cell lines. (A) Total RNAs probed with $tRNA^{Pro}$-derived oligonucleotide. (B) Poly A^+ RNAs probed with $tRNA^{Pro}$-derived oligonucleotide. (C) Poly A^+ RNA probed with HTLV-I LTR fragment. Arrows indicate the position of genomic RNA.

octadecanucleotides of various mammalian tRNAs (Table I) for use as hybridization probes to detect retroviral RNAs. These included the tRNAs used as primers for known retroviruses: $tRNA^{Pro}$ for HTLV-I,[2] HTLV-II,[2] human endogenous retroviruses[8,9] and mammalian type C retroviruses[2]; $tRNA^{Trp}$ for most avian type C retroviruses[2]; $tRNA^{Lys}_{CUU}$ for visna virus,[15] spuma retrovirus[16] and a human endogenous retrovirus[7]; $tRNA^{Lys}_{UUU}$ for HIV[2] and mouse mammary tumor virus[2]; $tRNA^{Glu}$, $tRNA^{Arg}$, $tRNA^{His}$ and $tRNA^{Ile}$ for human endogenous retroviruses[3-6] and $tRNA^{Phe}$ for intracisternal A particle.[17] Each oligonucleotide was labeled at the 5′-terminus with ^{32}P and used as a hybridization probe.

Detections of HTLV-I and HTLV-II RNAs with $tRNA^{Pro}$ probe Total cellular RNAs prepared from various human cell lines were separated by electrophoresis and hybridized with $tRNA^{Pro}$ probe (Fig. 1A). The RNAs from HTLV-I-infected cells (MT-2 and HUT 102) and HTLV-II-infected cells (Wil 2-43 and Ton 1) hybridized with this probe. (In the case of HTLV-II-infected cells, the expression of viral RNA was relatively poor and the hybridization signals appeared as smears.) However, ribosomal RNAs also hybridized with the same probe weakly and the hybridization pattern was not clear. Therefore, we selected poly A^+ RNA from these samples and retested them (Fig. 1B). With poly A^+ RNA, hybridized bands specific for HTLV-I-infected cells were very clear and these bands were not observed in T cells (Jurkat and H9), B cells (Wil 2 and Raji) or HeLa cells. To determine whether these bands were related to the HTLV-I sequence, we rehybridized the same membrane filter with HTLV-I LTR probe (Fig. 1 C). In addition to the bands seen in Fig. 1B, several new bands were observed, which must be those of subgenomic mRNAs for *env* and *pX*. As the primer binding site of HTLV-I is spliced out in the process of formation of these mRNAs,[18] $tRNA^{Pro}$ probe cannot hybridize with them (Fig. 1B). We concluded that the bands of MT-2 and HUT102 with the lowest mobility in Fig. 1B correspond to genomic RNA of HTLV-I judging from their size (~35S). The other bands with higher mobilities must be transcribed from defective proviruses present in the genomes of these cell lines.[19,20]

Poly A^+ RNA obtained from Wil 2-43, an HTLV-II infected cell line, gave the band corresponding to genomic RNA of HTLV-II with $tRNA^{Pro}$ probe (see Fig. 2A).

From these results we concluded that this method is applicable for detecting retroviral RNAs expressed in cells.

Search for retroviral RNAs expressed in various human cultured cells with $tRNA^{Pro}$ probe Poly A^+ RNAs prepared from colon cancer (SW-480), lung cancer (PC-13 and Lu-65), osteosarcoma (OST), bladder cancer (T-24), fibrosarcoma (HT-1080) and oral epidermoid cancer (KB) cells were analyzed with $tRNA^{Pro}$ probe. Wil 2 and Wil 2-43 (HTLV-II-infected Wil 2) cells were also analyzed as negative and positive controls, respectively. As shown in Fig. 2A, very strong hybridization was observed in Lu-65 cells as well as Wil 2-43 cells. The two major bands of Lu-65 were approximately 8.5 and

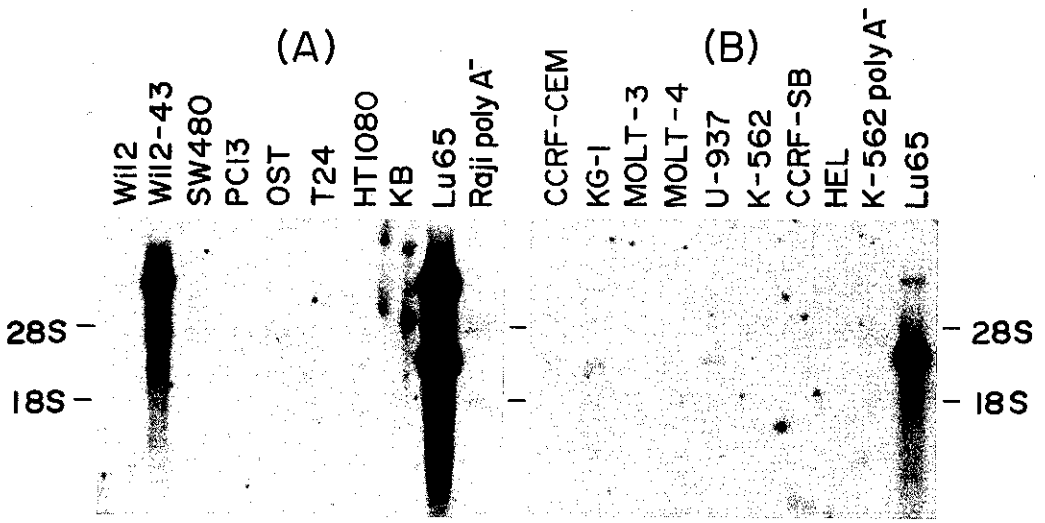

Fig. 2. Northern hybridization analysis of poly A^+ RNAs prepared from various cell lines probed with $tRNA^{Pro}$-derived oligonucleotide. (A) Solid tumor cell lines with negative (Wil 2) and positive (Wil 2-43) controls. (B) Leukemic cell lines with a positive control (Lu-65).

3.5 kilobases (kb) in size, which corresponded to the sizes of retroviral genomic RNA and *env* mRNA, respectively.

We also analyzed poly A^+ RNAs from various leukemic cell lines obtained from the Japanese Cancer Research Resources Bank (see "Materials and Methods"). Fig. 2B shows the results obtained with some of these cell lines. A weak hybridized band of about 3 kb was detected in CCRF-CEM, KG-1, MOLT-3 and U-937. Since these cell lines, except Lu-65, have never been maintained in mice, this band is not derived from *env* mRNA of murine leukemia virus (see below). This band was also observed in KY821, HLCL-1 and THP-1 (data not shown). When the filters shown in Fig. 1B and Fig. 2A were exposed for a longer period, this band was also seen in Jurkat, H9, Wil 2 and Raji, but not in cell lines established from solid tumors. We are now preparing cDNA clones of this RNA.

Sequence analysis of a cDNA clone obtained from Lu-65 cells To determine whether the hybridized bands of Lu-65 are related to retroviral RNA, we cloned Lu-65 cDNAs hybridized with $tRNA^{Pro}$ probe. One of the clones, named T3, which contains a 3.0 kb insert, was selected for sequence analysis. This insert was digested with *Eco*RI into fragments of 2.0 kb (T3L) and 1.0 kb (T3S). As T3S hybridized with $tRNA^{Pro}$ probe, we determined its nucleotide sequence. T3S is 962 base pairs (bp) long with a primer binding site for $tRNA^{Pro}$ and a long open reading frame (Fig. 3). Comparison with the sequences of known retroviruses showed a close resemblance of T3S with the 3′ *pol* and 5′ *env* regions of NZB xenotropic murine leukemia virus (MuLV).[21] Comparison of T3S with the NZB xenotropic MuLV genome showed correct splicing in T3S of all the *gag* sequence and most of the *pol* sequence. In the 962 bp analyzed, differences in only 24 nucleotides were observed, which would result in six amino acid substitutions in the *env* gene product (Fig. 3). The Lu-65 cell line was established from a human giant cell carcinoma of the lung transplanted into athymic nude mice (BALB/c, *nu/nu*),[22] so carcinoma cells may have been infected with BALB/c xenotropic MuLV during transplantation. Therefore, we prepared genomic DNAs from BALB/c mouse liver, Lu-65 cells and several human cultured cells and examined them by Southern hybridization with T3S probe. Lu-65 DNA gave several bands, whereas other human DNAs did not hybridize with this probe. On the other hand, mouse liver DNA gave very strong bands (data not shown). From these observations, we concluded that cDNA clone T3 was derived from the *env* mRNA of BALB/c xenotropic MuLV expressed in Lu-65 cells.

Screening with other tRNA probes Poly A^+ RNAs prepared from the various human cell lines listed in "Materials and Methods" were analyzed with the 16

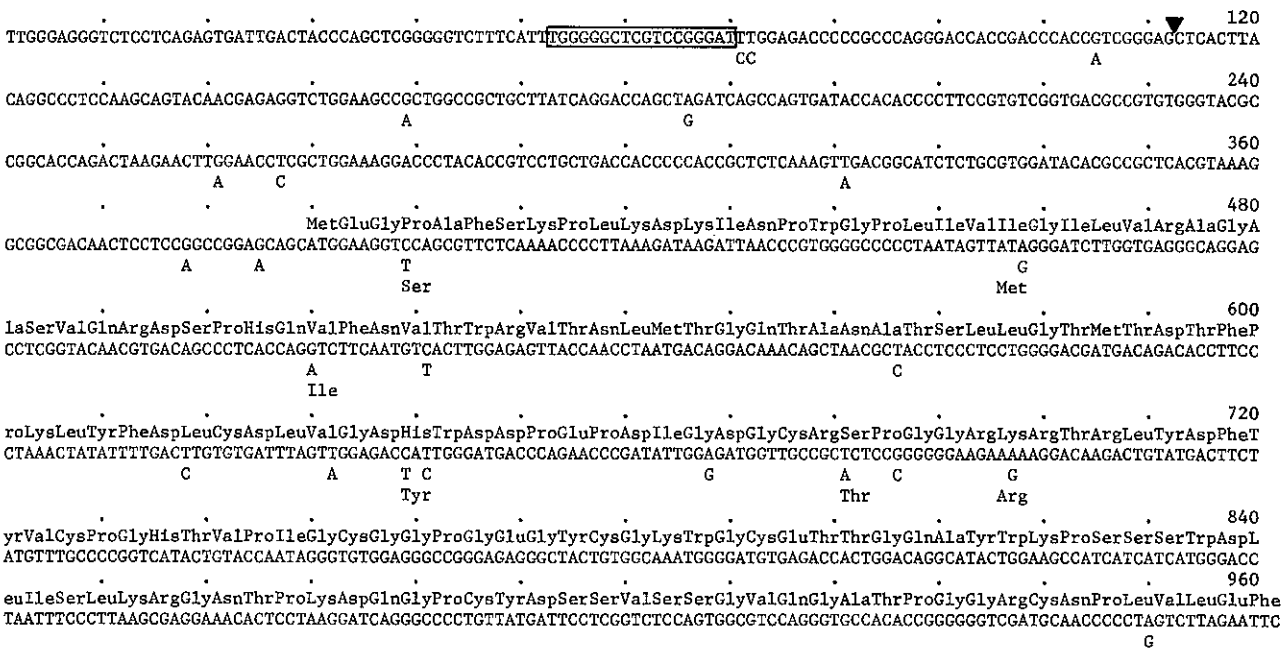

Fig. 3. Nucleotide sequence of T3S. The amino acid sequence of the *env* region is also shown. The primer binding site is boxed. The triangle indicates the position of splicing. Altered nucleotides and amino acids in NZB xenotropic MuLV are shown below the T3S nucleotide sequence.

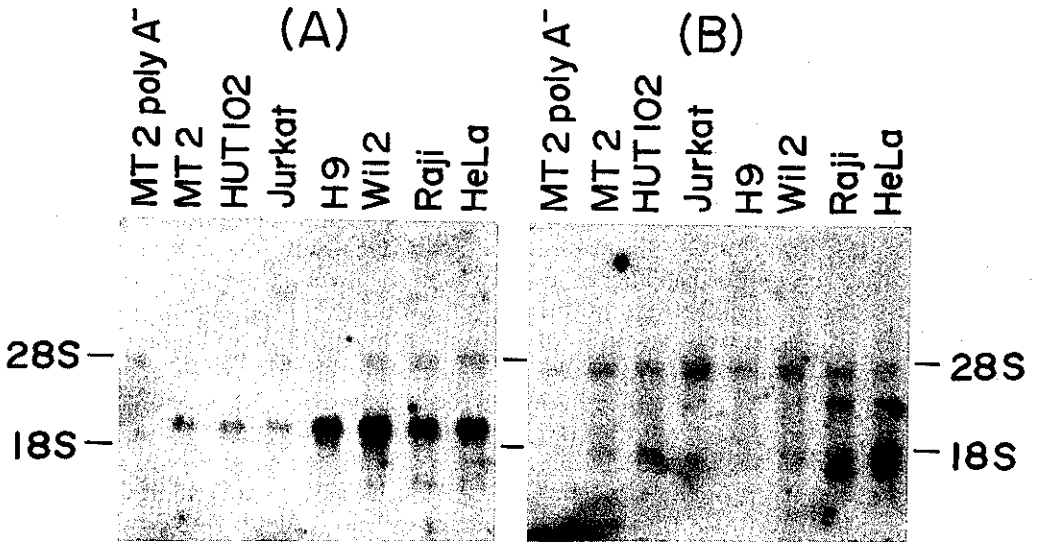

Fig. 4. Northern hybridization analysis of poly A^+ RNAs prepared from various cell lines probed with (A) $tRNA^{Gln}$- or (B) $tRNA^{Thr}$-derived oligonucleotide.

different tRNA probes other than $tRNA^{Pro}$ listed in Table I. $tRNA^{Lys}_{CUU}$, $tRNA^{Lys}_{UUU}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ile}$, $tRNA^{Gln}$ and $tRNA^{Thr}$ probes gave a specific band(s) ranging from 6 kb to less than 1 kb. The hybridization patterns with $tRNA^{Gln}$ and $tRNA^{Thr}$ probes are shown in Fig. 4. Some bands were observed in all cells, whereas others were detected only in certain cells. However, a typical hybridization pattern indicating retroviral infection, i.e. the presence of both genomic RNA (8–9 kb) and *env* mRNA (3–4 kb) as with Lu-65/$tRNA^{Pro}$ probe, was not observed in any case.

DISCUSSION

A very efficient method was developed for detecting expression of retroviral RNAs in cells. This method does not require any virus-specific sequence but only the 3′-terminal sequence of tRNA for detecting retroviral RNAs. By this method we could detect HTLV-I, HTLV-II and xenotropic MuLV RNAs in total RNA or poly A^+ RNA of virus-infected cells.

Although the 3′-terminal sequences of $tRNA^{Gln}$ and $tRNA^{Thr}$ differ in only two bases (Table I), the hybridization patterns obtained with these two tRNA probes were completely different (Fig. 4). This implies that the present method is specific enough to detect practically any particular RNA species.

Recently, Shih *et al.* used the polymerase chain reaction for detecting novel reverse transcriptase coding sequences in human nucleic acids.[23] Their method can detect retroviral RNA sequences, but it is limited to sequences resembling those of the primers used. In contrast, our method has no such limitation: it can detect retroviral RNA sequences containing the primer binding site with an appropriate tRNA probe.

We detected xenotropic MuLV RNAs in Lu-65 cells, which were established from human lung cancer cells transplanted into athymic nude mice. Lu-65 cells must have been infected with this murine endogenous retrovirus during transplantation. Therefore, care is necessary in using cell lines with such a history.

Thus our new method is very useful for searching for unidentified retroviruses expressed in human cancer cells. We are now examining RNAs prepared from various human cancer cell lines other than those used in this study and from various human cancer tissues.

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