Research Report

Application of Fluorescently Labeled Poly(dU) for Gene Expression Profiling on cDNA Microarrays

BioTechniques 31:406-413 (August 2001)

Edy Meiyanto, Jun-ichi Mineno[1], Norihiro Ishida, and Tatsuo Takeya
Nara Institute of Science and Technology, Ikoma, Nara, and [1]Takara Shuzo Ltd., Kusatsu, Shiga, Japan

ABSTRACT

The membrane filter hybridization technique has been widely used for gene expression profiling. The preparation of sensitive and reliable probes is critical for quantitative analysis in this technique. We report a method in which fluorescently labeled poly(dU) is used to detect poly(A)-containing mRNA that hybridizes to specific gene targets. The probe can be used commonly for every sample, alleviating problems encountered in preparing cDNA probes by reverse transcription, particularly when many samples are to be analyzed. Moreover, the sensitivity is at least comparable to cDNA probes prepared by conventional protocols, and intensities of signals after hybridization are independent of mRNA sizes and solely dependent on copy numbers. This method was also shown to be applicable to DNA chip technology.

INTRODUCTION

The membrane filter hybridization technique has been widely used to identify species and/or to quantify levels of mRNA expressed in cells (1). One application method is to immobilize either total RNA or purified mRNA on membranes and to incubate with gene-specific probes labeled with either fluorescent or radioactive nucleotides; northern blot analysis belongs to this category. In other contrasting applications, gene-specific polynucleotides are fixed on membranes and hybridized with cDNA that are prepared and labeled from a mixture of mRNA-containing sequences to be detected; dot blotting is an example. These two methods have been used in a variety of studies. More recently, the technique was refined and developed in a cDNA microarray system in which high-density cDNA analysis became possible by the use of a glass surface (2,5,8,21). In all methods, the preparation of sensitive and reliable probes is essential, preferably using simple procedures.

Here, we report a method in which fluorescently labeled poly(dU) was used to detect poly(A)-containing mRNA that hybridized to specific gene targets. Because the poly(dU) probe can be applicable to all eukaryotic samples, this method alleviates many problems encountered when preparing cDNA probes for each sample. Moreover, the signal intensities after hybridization are independent of mRNA sizes and solely dependent on copy numbers, which provide a great advantage when comparing relative expression levels of genes. This method was further shown to be applicable to DNA chip technology. In our study, to comply with the terms commonly used in hybridization technologies, "probes" refers to the DNA molecules in the hybridization solution, while "targets" refers to the DNA molecules immobilized on either the membrane or glass surface.

MATERIALS AND METHODS

Deoxyribonucleotides and Polynucleotides

Deoxyribonucleotides and synthetic polynucleotides including poly(rA) and $p(dT)_{12-18}$ were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Cy3-dUTP and Cy5-dUTP were also purchased from Amersham Pharmacia Biotech, and Alexa-546-14-dUTP was obtained from Molecular Probes (Eugene, OR, USA).

Preparation of Fluorescently Labeled Poly(dU) Probe

Labeling reactions were performed by reverse transcription (RT) in the presence of 100 pmol poly(rA)·$p(dT)_{12-18}$; 0.5 mM each dATP, dCTP, and dGTP; 40 µM dTTP; 20 µM fluorescently labeled dUTP; 10 mM dithiothreitol (DTT); 0.5 U/µL ribonuclease inhibitor (Life Technologies, Rockville, MD, USA); and 0.03 U/µL SUPERSCRIPT™ reverse transcriptase (Life

Technologies) in a 50-µL solution. Reaction mixtures were incubated at 30°C for 60 min, followed by heat treatment for 3 min at 94°C and chilling on ice. The purified products through Microspin S-300HR columns (Amersham Pharmacia Biotech) were treated with 0.3 N NaOH for 20 min at 37°C to degrade the template poly(rA), followed by neutralization with HCl and Tris-HCl, pH 7.0, to a final volume of approximately 100 µL.

Cell Culture, RNA Extraction, RT-PCR, and cDNA Probe Preparation

Human HeLa and WI38 cells and RAW247 mouse monocyte/macrophage line cells were obtained from the Human Science Research Resources Bank (Osaka, Japan) and maintained in DMEM supplemented with 10% FCS. Differentiation of RAW247 cells to osteoclasts was induced with osteoclast differentiation factor stimulation (15) (unpublished data). Total RNA was extracted using the acid guanidinium-phenol chloroform procedure from cultured cells, reverse transcribed into cDNA, and used for RT-PCR; under the conditions used, a linear amplification was achieved (16). Poly(A)-containing RNA was purified using QuickPrep™ mRNA purification kit (Amersham Pharmacia Biotech) when necessary.

cDNA probes were prepared by RT as previously described for labeling poly(dU), except that 600 ng purified mRNA and Cy-3 dUTP were used instead of poly(rA) and Alexa-dUTP, respectively. Reaction products were finally resuspended with 10 µL hybridization solution.

Gene-Specific Targets

Unique regions of human GAPDH (6), PCNA (24), p53 (18), Rb (23), Ras (11), p130 (12), p14 (9), p16 (9), p18 (22), p19 (10), p21 (13), and p27 (20) were amplified from human cells as previously described and immobilized on membranes (Hybond®+) using a Bio-Dot™ (Bio-Rad Laboratories, Hercules, CA, USA). IntelliGene™ Test Array (Takara Shuzo, Shiga, Japan) was a DNA chip containing a duplicated set of 48 human and 48 cyanobacterium target genes on a glass surface. IntelliGene Mouse CHIP (Takara Shuzo) contained mouse genes.

Hybridization and Monitoring

Membrane filters carrying target DNA were prehybridized in 1 mL hybridization solution containing 5× standard saline citrate (SSC), 0.5% BSA, 0.1 mg/mL salmon sperm DNA, and 0.1% SDS/cm^2 of membrane at 30°C for 1 h in a bag. Either total RNA or purified mRNA was heated together with 10 µL poly(dU) probe solution in 200 µL hybridization buffer at 70°C for 10

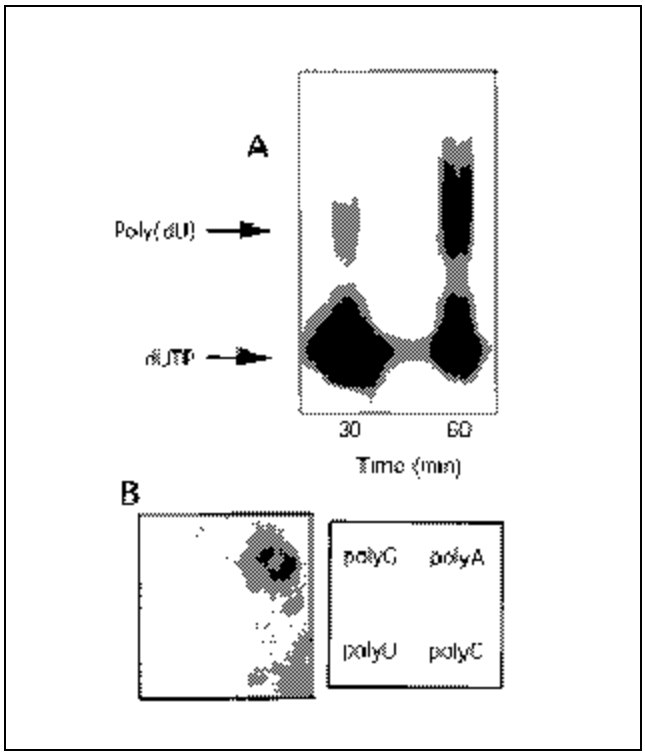

Figure 1. Preparation and specificity of the poly(dU) probe. RT reaction was carried out at 30°C in the presence of poly(rA)-p(dT)$_{12-18}$. Reaction products at 30 and 60 min, respectively, were examined by agarose gel electrophoresis. The positions of the reaction products and free fluorescent dUTP are indicated on the left. (B) The poly(dU) probe was hybridized with a membrane on which poly(rA), poly(rC), poly(rG), and poly(rU) had been immobilized. Signals detected after hybridization with the probe are shown in the top panel, and the positions of each polynucleotide are shown in the bottom panel.

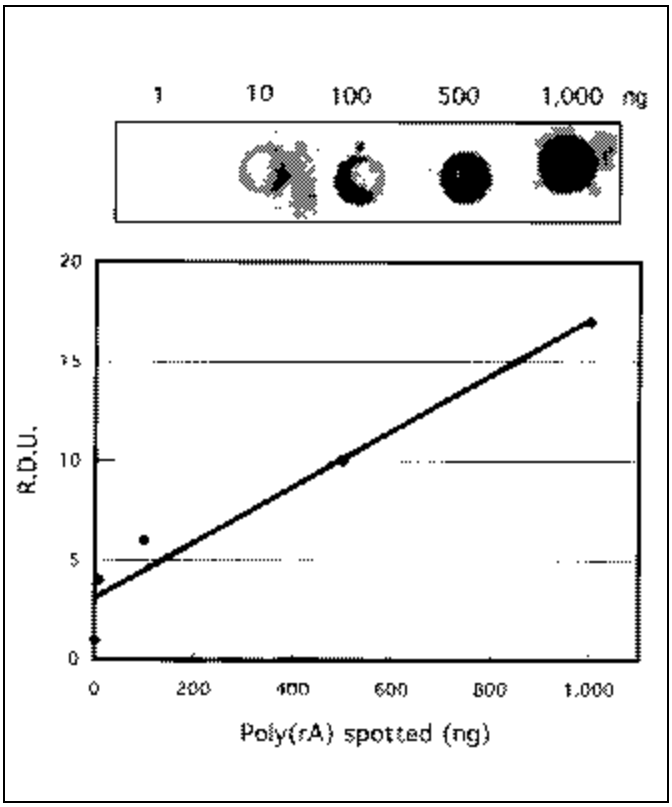

Figure 2. Sensitivity test of the poly(dU) probe using poly(rA). Poly(rA), at the indicated amounts in the top panel, was spotted on nylon membranes and then hybridized with the poly(dU) probe as described in Materials and Methods. Relative densitometry units (R.D.U.) were normalized to the 1-ng signal and are shown in the bottom panel. Microsoft® Excel® was used for graphical drawing and correlation coefficient determination; the r value was 0.95.

min, transferred into the bag, and then hybridized with the membrane for 6–10 h at 30°C. The membrane was washed for 15 min at room temperature in low-stringency washing buffer (1× SSC and 0.1% SDS), then in high-stringency washing buffer (0.1× SSC and 0.1% SDS) for 10 min. Membranes were monitored using a *FM*BIO® II (Hitachi Software, Japan) fluorescent scanning apparatus.

Hybridization with DNA chips was carried out according to the manufacturer's instructions. Briefly, for the poly(dU) probe hybridization, 600 ng or 3 µg mRNA were preheated together with 10 µL poly(dU) probe (14 µL total) at 70°C for 10 min. Chips were incubated with 14 µL prehybridization solution (6× SSC, 0.1% SDS, 5× Denhardt's solution, and 0.1 µg/µL denatured salmon sperm DNA) containing either poly(dU) probe or cDNA probe at 65°C for 18–20 h, followed by 3 h incubation at 30°C. Chips were washed for 15 min at 60°C in 2× SSC and for 15 min at 60°C in solution containing 2× SSC and 0.1% SDS and rinsed twice with 0.2× SSC at room temperature. Hybridization profiles were analyzed using an Affymetrix 418 array scanner (Affymetrix, Santa Clara, CA, USA).

RESULTS

Preparation of the Fluorescently Labeled Poly(dU) Probe and Its Characterization

Fluorescently labeled poly(dU) was prepared by RT reactions on poly(rA) in the presence of Alexa-dUTP. The optimal incorporation of the substrates was obtained when the ratio of dUTP and dTTP was 1:2, the mean size of the products was approximately 800 bases, and the poly(rA) size used as a template was between 300 and 1500 bases (Figure 1A). The binding ability and specificity of the poly(dU) probe thus prepared have been examined and shown to hybridize only to poly(rA) spotted on membrane filter (Figure 1B).

The sensitivity was examined within a range of concentrations of fmol (ng) to pmol (µg) of poly(rA) as a target. When as few as 3 fmol poly(rA) were fixed on a 12-mm^2 (area) dot (diameter: 4 mm), the signal was clearly detected (Figure 2, top panel), and the intensity of each spot increased in a linear manner within a range of 10–1000 ng of the target (Figure 2, bottom panel).

Application to Profiling of Gene Expression on Filter Membranes

This new method was used to examine mRNA levels of certain genes in human cells. First, when total RNA from HeLa cells was hybridized with

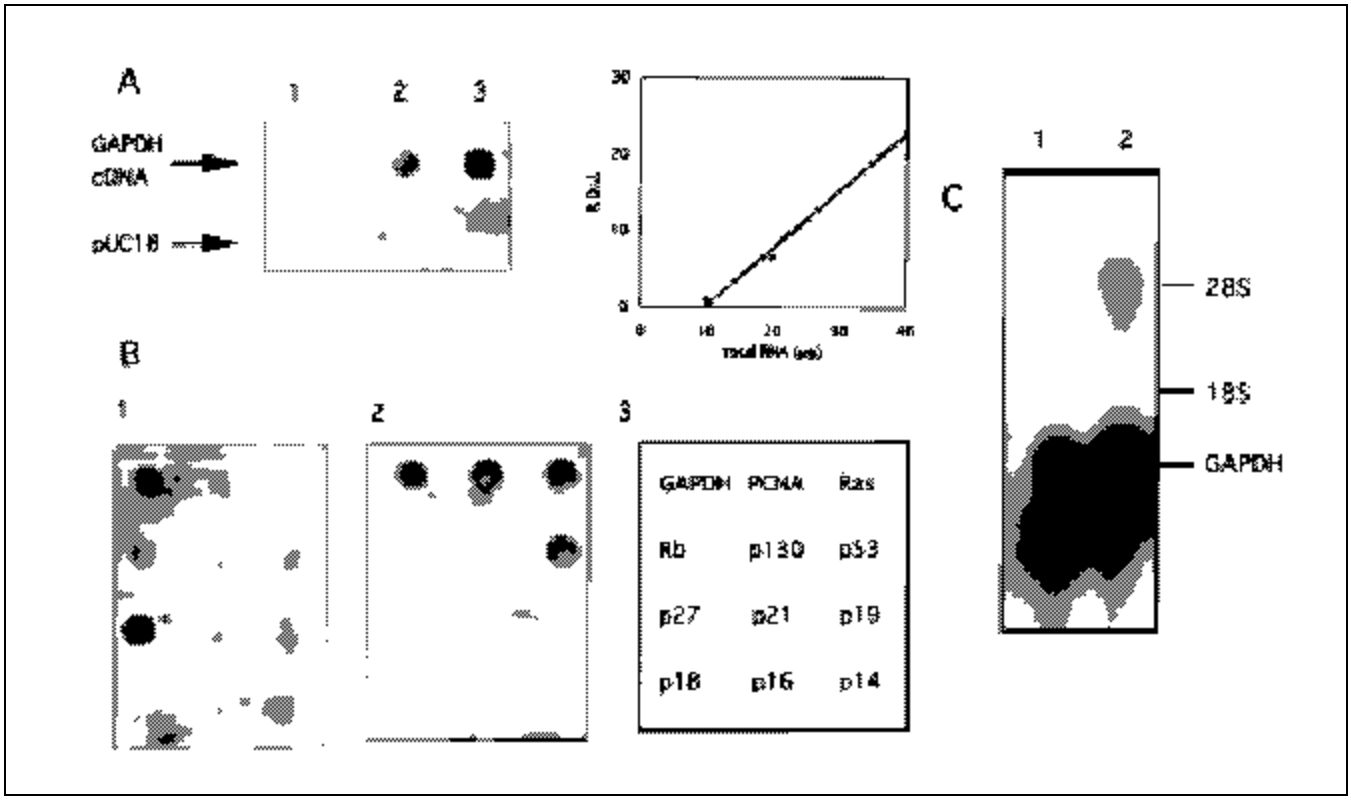

Figure 3. Monitoring genes expressed in human cells. (A) One microgram of GAPDH DNA (top) and pUC18 DNA (bottom) were spotted on the nylon membrane and hybridized with increasing amounts of total RNA from WI-38 cells: (no. 1) 10, (no. 2) 20, and (no. 3) 40 µg (left panel). Total RNA was labeled with the poly(dU) probe as described in Materials and Methods. Densities were normalized to the 10-µg signal and are shown in the right panel; the r value was 0.95. (B) Differential gene expression profiles between WI-38 cells and HeLa cells. Twelve cDNA clones of human genes (1 µg each) including growth- and cell cycle-regulated genes were spotted on the nylon membranes and probed with 20 µg total RNA from (no. 1) WI-38 cells and (no. 2) HeLa cells. (no. 3) shows the species and positions of the spotted cDNA clones. (C) Northern blot analysis of GAPDH mRNA from (no. 1) WI-38 and (no. 2) HeLa cells. Arrows indicate the positions of 28S and 18S rRNA, respectively.

GAPDH cDNA and the control plasmid pUC18, signals were detected only by the GAPDH target and not by the pUC18 DNA (Figure 3A), indicating that this probe could label poly(A)-containing mRNA under the conditions used. Furthermore, the intensities of signals obtained increased in a linear manner (Figure 3A), indicating that total RNA could hybridize quantitatively at least within this range.

Then, the feasibility of this method for profiling gene expression was examined using two types of human cell lines. WI-38 is a diploid cell line derived from normal embryonic lung tissue (14), and HeLa is a tumor cell line derived from cervical cancer. Figure 3B shows the findings obtained from WI-38 cells in the confluent condition and proliferating HeLa cells, respectively. The integrity of the mRNA prepared was confirmed by northern blot analysis of GAPDH mRNA (Figure 3C). Except for GAPDH mRNA, which was commonly detected in both cell types, the expression profiles of other genes were not identical between WI-38 and HeLa cells, reflecting the differences in their original tissues and/or physiological conditions. The expression of the p53 gene and low expressions of the p21 gene in HeLa cells have been reported (4). However, low expression of PCNA and high expression of p27 mRNA were prominent in growth-arrested WI-38 cells.

Application to DNA Chips

Since this method has been shown to be applicable on membrane filters, we examined the possibility of its application to DNA chips. We used the Test Array as a model chip and examined mRNA from WI-38 cells. This array contained a duplicated set of 96 target DNA clones (each target was numbered from I to VIII for rows and from 1 to 12 for columns in Figure 4A): 48 from human cells (columns 1–6) and 48 from cyanobacterium (columns 7–12). As shown in Figure 4A, WI-38 mRNA was shown to hybridize to selected targets from human sources, while signals were completely absent in cyanobacterium genes, confirming its applicability to DNA chips. However, some targets gave signals using the poly(dU) probe alone (Figure 4B; targets I-4 and IV-3), suggesting the existence of poly(dA) tracts in these targets.

The same chip was then hybridized with a cDNA probe prepared by a conventional method from the same mRNA preparation, resulting in the profile shown in Figure 4C. Except for the two above targets (I-4 and IV-3), signals detected with two probes could be classified into three groups: the first one contained common signals to both probes (e.g., IV-1, V-1, V-2, and VI-4), the second one was unique to the poly(dU) probe (e.g., VI-3), and the third one was unique to the cDNA probe (e.g., VII-5).

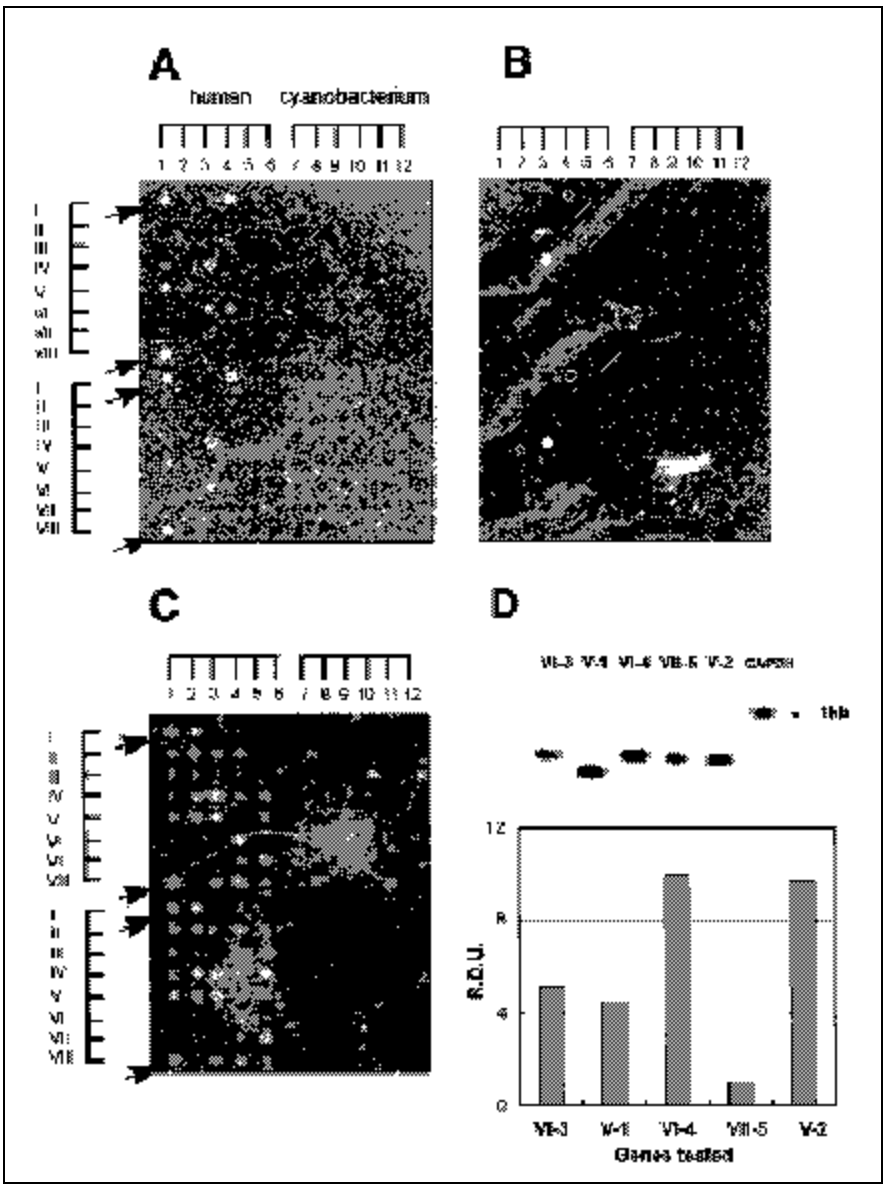

Figure 4. Monitoring gene expression profiles on DNA chips. (A and B) The poly(dU) probe and cDNA probe prepared by a conventional method (C), respectively, were used to monitor gene expression profiles on the test array chip. Each contains a duplicated set of 96 target DNA clones originating from 48 human genes (left half) and 48 cyanobacterium genes (right half). mRNA (600 ng) from WI-38 cells were used in panels A and C, respectively; no cellular RNA was included in panel B. Each spot is represented by a row number (roman numerals) and column number (arabic numerals). Arrows indicate the position of β-actin cDNA (I-1 and VIII-1). (D) Verification of signals detected in panels A and C. Specific primers for target genes [V-1 (7), V-2 (25), VI-3 (19), VI-4 (26), and VII-5 (3)] including GAPDH were designed and their expression levels in WI-38 cells were examined by RT-PCR. Profiles of the products separated on 1% agarose gel are shown (top panel). Their densities were quantified, normalized to the VII-5 signal, and are shown (bottom panel).

To verify the accuracy of the newly developed method, we chose several target genes and examined their expression levels in WI-38 cells by RT-PCR (Figure 4D): V-1, cysteine protease Mch2 isoform (7); V-2, laminin-binding protein (25); VI-3, CD36 (19); VI-4, MDC9 (26); and VII-5, CDC37 (3); in addition to GAPDH as a positive control. VI-3, VI-4, V-1, and V-2 were found to be expressed, while the expression level of VII-5 was significantly lower compared with the other four tested targets (Figure 4D). Taken together, the new labeling method could give fairly reliable results as tested.

Preparation of Differentially Labeled Poly(dU) Probes and their Applications

One prevailing application of DNA chip technology is to detect genes expressed differentially between cells in different physiological conditions or stages. To confirm that the new method could also be applicable to this purpose, we prepared Cy3-labeled and Cy5-labeled poly(dU) probes, respectively, and applied them to gene expression profiling in differentiating cells. mRNA prepared from progenitor RAW247 cells were labeled with the Cy3-poly(dU) probe and mRNA from mature osteoclasts were labeled with the Cy5-poly(dU) probe, respectively, and hybridized with DNA chips containing mouse genes. As shown in Figure 5, genes specifically expressed in either RAW247 cells or in osteoclasts were detected distinctively.

DISCUSSION

In the present study, we showed that the poly(dU) product could be used to detect mRNA species hybridized on DNA arrays spotted on either membranes or glass surfaces (DNA chips). There are two main advantages of the poly(dU) probe over the conventional cDNA probe. First, the intensities given by the probe are solely dependent on copy numbers of the respective mRNA species regardless of their sizes. Second, it is unnecessary to carry out the RT reaction for cDNA preparation each time, once the probe is prepared and the probe can be commonly used.

To verify such advantages, we examined the gene expression profiles of WI-38 cells using DNA chips and probes prepared by two different methods: the poly(dU) probe and a cDNA probe prepared by a conventional method (Figure 4). Although they shared common profiles on some targets, such as the β-actin gene (Figure 4, A–C), the profiles given by the two probes were not identical. The expression of genes detected with the poly(dU) probe was subsequently confirmed by RT-PCR. However, a gene detected only with the cDNA probe turned out to be expressed at a low level (Figure 4D). This difference could be caused by the characteristic property of each probe. The poly(dU) probe anneals solely with the poly(A) region of mRNA irrespective of their sizes, as described above. However, the original sizes of mRNA are not homogeneous; moreover, RT reactions can be hampered by higher structures of template mRNA in the process of cDNA preparation, causing further heterogeneities in the lengths of the cDNA products. Therefore, the intensities of the signals after hybridization by cDNA probes could be severely affected by such factors.

It is essential for any detection method to discriminate real signals from false or artificial ones. In this respect, the possible existence of either a repetitive sequence consisting of poly(dA) in the untranslated region or a poly(A)-tail sequence of the target cDNA clones on chips cannot be overlooked in the method using the poly(dU) probe. In fact, Figure 4B shows such an example in which two targets (I-4 and IV-3) gave signals in the absence of cellular RNA, suggesting that they contained either one of the two forms of the poly(dA) tracts in their sequences. These two spots were also detected in Figure 4A, where additional signals appeared in the presence of mRNA. Taken together, we confirmed that the detection method described here possessed several advantages for microarray analysis. However, if target DNAs contain poly(dA) tracts, then the method could cause noise and require that some precautions be taken in data analysis.

The length of the poly(rA) residues at the 3′ termini of mRNA varies between 200 and 250 nucleotides (17). However, the length is not heterogeneous in each mRNA. Therefore, if

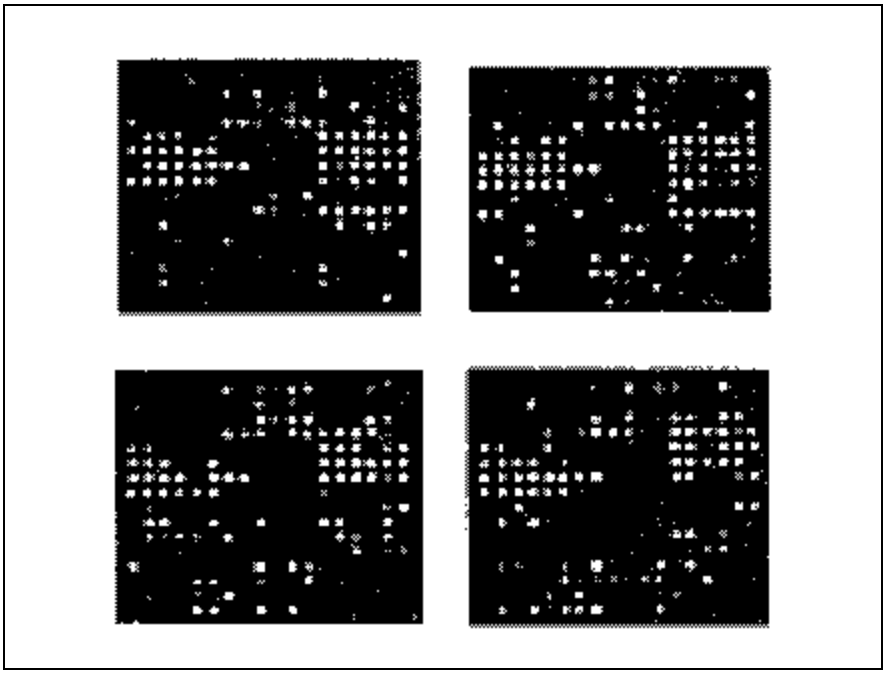

Figure 5. Gene expression profiling with differentially labeled poly(dU) probes. mRNA (3 μg) prepared from progenitor RAW247 cells and mature osteoclasts were labeled with Cy3-labeled (red) and Cy5-labeled (green) poly(dU) probes, respectively, and hybridized with DNA chips containing mouse cDNAs as targets. This chip consists of four different meta-grids.

poly(dU) with approximately 200 bases or longer were used as a probe, then the length polymorphism would not cause a significant effect on quantification of the expression levels. Expression of certain genes is also known to be regulated by either poly- or de-adenylation at their 3′ termini (17). This type of regulation could be detected if the poly(dU) probe is used in combination with cDNA probes.

Finally, two kinds of cDNA probes labeled with different fluoresceins, such as Cy-3 and Cy-5, have been used to detect the changes in expression of mRNA under two different conditions. This technique can also be applicable to the poly(dU) probes; namely, two kinds of poly(dU) probes differentially labeled can easily be prepared and used. This was confirmed by applying the method to detect genes differentially expressed through osteoclatogenesis in vitro (Figure 5).

REFERENCES

1. **Asubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seiman, J.A. Smith, and K. Struhl.** 1994. Current Protocols in Molecular Biology, Greene, Toronto.
2. **Brown, P.O. and D. Botstein.** 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. Suppl. *21*:33-37.
3. **Dai, K., R. Kobayashi, and D. Beach.** 1996. Physical interaction of mammalian CDC37 with CDK4. J. Biol. Chem. *271*:22030-22034.
4. **Desaintes, C., S. Goyat, S. Garbay, M. Yaniv, and F. Thierry.** 1999. Papillomavirus E2 induces p53-independent apoptosis in HeLa cells. Oncogene *18*:4538-4545.
5. **Duggan, D.J., M. Bittner, Y. Chen, P. Meltzer, and J.M. Trent.** 1999. Expression profiling using cDNA microarrays. Nat. Genet. Suppl. *21*:10-14.
6. **Ercolani, L., B. Florence, M. Dernaro, and M. Alexander.** 1988. Isolation and complete sequence of functional human glyceraldehyde-3-phosphate dehydrogenase gene. J. Biol. Chem. *263*:15335-15341.
7. **Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri.** 1995. Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res. *55*:2737-2742.
8. **Gerhold, D., T. Rushmore, and C.T. Caskey.** 1999. DNA chips: promising toys have become powerful tools. Trends Biochem. Sci. *24*:168-173.
9. **Guan, K.L., C.W. Jenkins, Y. Li, M.A. Nichols, X. Wu, C.L. O'Keefe, A.G. Matera, and Y. Xiong.** 1994. Growth suppression by p18, a $p16^{INK4/MTS1}$- and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlated with wild-type pRb function. Genes Dev. *8*:2939-2952.
10. **Guan, K.L., C.W. Jenkins, Y. Li, C.L. O'Keefe, S. Noh, M. Zariwala, A.G. Matera, and Y. Xiong.** 1996. Isolation and characterization of $p19^{INK4d}$, a p16-related inhibitor specific to CDK4 and CDK6. Mol. Biol. Cell *7*:57-70.
11. **Hall, A. and R. Brown.** 1985. Human N-ras: cDNA cloning and gene structure. Nucleic Acids Res. *13*:5255-5268.
12. **Hannon, G.J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb related p130 through its interaction with CDK and cyclins. Genes Dev. *7*:2378-2391.
13. **Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge.** 1993. The p21 CDK-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*:805-816.
14. **Hayflick, L. and P.S. Moorhead.** 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*:585-621.
15. **Hsu, H., D.L. Lacey, C.R. Dunstan, I. Soloyev, A. Colombero, E. Timms, H-L. Tan, G. Elliott et al.** 1999. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoproteogerin ligand. Proc. Natl. Acad. Sci. USA *96*:3540-3545.
16. **Itahana, K., Y. Morikazu, and T. Takeya.** 1996. Differential expression of four connexin genes, Cx-26, Cx-30.3, Cx-32, and Cx-43, in the porcine ovarian follicle. Endocrinology *137*:5036-5044.
17. **Jackson, R.J. and N. Standart.** 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell *62*:15-24.
18. **Matlashewski, G., P. Lamb, D. Pim, J. Peacock, L. Crawford, and S. Benchimol.** 1984. Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. EMBO J. *3*:3257-3262.
19. **Oquendo, P., E. Hundt, J. Lawler, and B. Seed.** 1989. CD36 directly mediates cytoadherence of *Plasmodium falciparum* Cell *58*:95-101.
20. **Polyak, A., M.H. Lee, H. Erdjument-Bromage, A. Koff, J.M. Roberts, P. Tempest, and J. Massague.** 1994. Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*:59-66.
21. **Schena, M., D. Shalon, R.W. Davis, and P.O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science *270*:467-470.
22. **Serrano, M., G.J. Hannon, and D. Beach.** 1993. A new regulatory motif in cell-cycle control causing inhibition of cyclin D/CDK4. Nature *366*:704-707.
23. **Toguchida, J., T.L. MecGee, J.C. Paterson, J.R. Eagle, S. Tucker, D.W. Yandel, and T.P. Dryja.** 1993. Complete genomic sequence of the human retinoblastoma susceptibility gene. Genomics *17*:535-543.
24. **Traveli, S., D.H. Ku, M.G. Rizzo, L. Ottavio, R. Baserga, and B. Calabretta.** 1989. Structure of the human gene for the proliferating cell nuclear antigen. J. Biol. Chem. *264*:7466-7472.
25. **Van den Ouweland, A.M., H.L. Van Duijnhoven, K.A. Deichmann, J.J. Van Groningen, L. de Leij, and W.J. Van de Ven.** 1989. Characterization of a multicopy gene family predominantly consisting of processed pseudogenes. Nucleic Acids Res. *17*:3829-3843.
26. **Weskamp, G., J. Kratzscmar, M.S. Reid, and C.P. Blobel.** 1996. MDC9, a widely expressed cellular disintegrin containing cytoplasmic h3 ligand domains. J. Cell Biol. *132*:717-726.

Received 10 July 2000; accepted 22 January 2001.

Address correspondence to:

Dr. Tatsuo Takeya
Graduate School of Biological Sciences
Nara Institute of Science and Technology
Ikoma, Nara 630-0101, Japan
e-mail: ttakeya@bs.aist-nara.ac.jp