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A Simple and Useful Method for Simultaneous Screening of Elevated Levels of Expression of a Variety of Oncogenes in Malignant Cells

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Abnormal expression of various oncogenes has been implicated in the development of many malignant tumors. Although RNA blotting methods have been used to measure abnormal expression, they involve the time-consuming process of individually labeling the oncogene probes. To simplify this process we have attempted to develop a new method, termed simultaneous screening, which is based on the synthesis of radiolabeled cDNA corresponding to the mRNA population of malignant cells and on hybridization with various oncogene probes, immobilized on a membrane filter. This method circumvents the time-consuming process of the prevailing RNA blotting methods and is also sensitive enough to detect accurately a five- to ten-fold level of expression of rare mRNA (~10 copies per cell). Overexpression of ten oncogenes was detected in a variety of malignant cells and mitogen-stimulated cells with this method. These results suggest that our simultaneous screening method can be used to examine the overexpression of oncogenes.

Key words: Oncogene — Oncogene expression — Simultaneous screening method — cDNA

More than forty oncogenes have been detected and isolated in the past several years.[1] Studies on the relationship between abnormal expression of these oncogenes and variations in the clinical behavior of malignant tumors have indicated that elevated levels of expression may be involved in the carcinogenesis of a wide range of malignant tumors[2–5]; for example, c-*myc* expression in a series of leukemias[2] and N-*myc* expression in neuroblastoma.[5] Investigation of oncogene(s) expression in a variety of human malignancies will extend our understanding of the malignant properties and the process of carcinogenesis at the molecular level.

It is a time-consuming and complicated process, however, to measure individually the expression of all oncogenes present by Northern or RNA dot blot hybridization.[6, 7] This is because the number of oncogene probes available has been increasing rapidly, and a rehybridization process is required to detect each oncogene expression.

In order to simplify the measurement of oncogene expression, we attempted to detect simultaneously the expression of various oncogenes with radiolabeled cDNA[*3] probes synthesized from the objective cells. This study reports in detail the procedure involved in the method, termed the simultaneous screening method, and the detection of overexpression of ten oncogenes in malignant cells and mitogen-stimulated cells.

Materials and Methods

Cells and Tumor Specimens The cells used in this study were 11 human tumor cell lines, one avian tumor cell line, NIH/3T3 cells transformed by two cloned oncogenes, Syrian hamster (SHOK) cells transformed by X-ray-induced mouse osteosarcoma DNA and human thyroid cancer DNA, and Balb/3T3 cells. Primary human fibroblasts, NIH/3T3 and SHOK cells were also used as negative controls.

Tumors were obtained at surgical operations performed in the second Department of Surgery, Osaka University Medical School. The tumors studied were six colorectal cancers and four other malignant tumors.

RNA Preparation and Blotting Analysis Total RNA was prepared by the guanidinium/CsCl

[*3] Abbreviations: cDNA, complementary DNA; poly(A)$^+$ RNA, polyadenilated RNA; dNTP, deoxyribonucleoside triphosphate.

method.[8] Poly(A)$^+$ RNA were purified with m-AP paper[9] (Takara, Kyoto) as suggested by the manufacturers.

For RNA dot blotting analysis, 8 μg of total RNA per sample was diluted serially (2:1) and then spotted on a nylon membrane, Hybond N (Amersham). For Northern blotting analysis, 20 μg of total RNA per sample was denatured with formaldehyde and electrophoresed through 1% agarose-formaldehyde gel,[6] and then transferred to the Hybond N membrane.

Synthesis of Radiolabeled cDNA Probes and the Simultaneous Screening Method cDNA probes were labeled in a 50 μl reaction mixture of 50m*M* Tris-HCl (pH 8.3), 10m*M* $MgCl_2$, 100m*M* KCl, 1 m*M* dTTP, dATP, dGTP, 40 μM [α-^{32}P]dCTP (29 Ci/mmol, 10 μCi/μl; 33004, New England Nuclear; NEN), 0.4m*M* dithiothreitol, 100 μg/ml, oligo[dT], 50 μg total RNA, and 20 units of avian myeloblastic leukemia virus reverse transcriptase (Takara), and then incubated at 42° for 1.5 hr, unless otherwise indicated. The reaction was terminated by adding 4 μl of 0.5*M* EDTA and 25 μl of 0.1*N* NaOH. The mixture was incubated at 65° for one hour and then neutralized by adding 25 μl of 1*M* Tris-HCl (pH 7.5) and 1*N* HCl, respectively. After phenol/chloroform extraction, the mixture was passed through a G-50 Sephadex column. The eluted mixture was then denatured at 100° for 5 min, chilled on ice and hybridized to a nylon filter with an excess amount (5–10 μg) of twenty oncogene plasmid DNAs immobilized on it. The quality of cDNA probes was monitored by immobilizing three or five human β-actin subcloned DNAs containing different coding regions, used as positive controls and pBR322 plasmid DNA, as a negative control (see "Results and Discussion"). Hybridization was performed for 36–48 hr at 65° in 6×SSC, 0.1% SDS, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin, 100 μg/ml denatured salmon sperm DNA, and labeled cDNA probes (about 1–2×10^7 cpm/ml). The filter was washed for 2×60 min with 2×SSC, 0.1% SDS at 65° and then for 60 min with 0.2–0.5×SSC, 0.1% SDS at 65°. After autoradiography, the intensity of each spot was determined by densitometric scanning using a DU-8 spectrometer (Beckman).

Alkaline Agarose Gel Electrophoresis The length of the cDNA probes was determined by alkaline agarose gel electrophoresis.[10] Phage λ DNA, digested with *Hin*dIII, was used as a length marker.

Results and Discussion

Reaction Conditions for Synthesis of cDNA Probes To develop a simple and effective procedure for the synthesis of the hybridization cDNA probes, we investigated the optimal conditions by altering the concentration of the unlabeled dCTP, the specific activity of the labeled dCTP, and the purity of the template RNA. HL60 cells were examined as a source for the template RNA because amplification and overexpression of c-*myc* were reported in these cells.[2, 11] Salt concentration, incubation time and temperature, and concentrations of dNTPs except dCTP, have been shown to regulate optimal synthesis of cDNA.[12] Labeled dCTP was commercially obtained and used without any treatment to simplify the process.

Table I shows that condition A produced the most favorable results with respect to both the yield and the specific activity of total cDNA probes. Conditions A and D produced the most favorable cDNA probe lengths, whereas with conditions B and C the average

Table I. Comparison of Reaction Conditions for cDNA Probe Synthesis

Condition	Template RNA[a]	Specific activity of [α-^{32}P]dCTP (Ci/mmol)	Concentration of dCTP (μM)		Yield[b] (%)	Specific activity of total cDNA probes[c] (cpm)
			[α-^{32}P]dCTP	unlabeled dCTP		
A	50 μg total RNA	28.9	40	0	16.7	8.3×10^7
B	Poly(A)$^+$ RNA purified from 50 μg total RNA	28.9	40	0	9.6	2.5×10^7
C	50 μg total RNA	3703	0.3	0	0.2	9.8×10^9
D	50 μg total RNA	3703	0.3	1000	28.5	1.5×10^7

a) The source of template RNA was HL60 cells.

b) Yield is indicated in relation to input poly(A)$^+$ RNA, estimated from incorporated [α-^{32}P]dCTP, assuming that total RNAs contain 2% mRNAs.

c) Determined after fractionation on a Sephadex G-50 column.

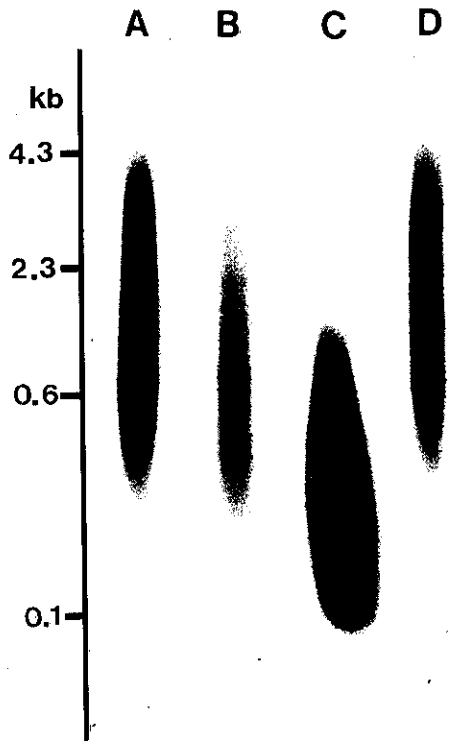

Fig. 1. Size of cDNA probes synthesized using four different conditions as listed in Table I. The size of the cDNA probes was analyzed on a 1% alkaline agarose gel. Lane A, condition A; lane B, condition B; lane C, condition C; lane D, condition D. The molecular size scale is in kilobases.

Table II. Expression Level and Spot Intensity of H-*ras* in NIH/3T3 Cells and Transformants by EJ-*ras*

Cell line	Relative level[a] of H-*ras* expression	Relative spot intensity of H-*ras* to β-actin[b] (%)
NIH/3T3-Rf-2-2[c]	22	12.6, 9.0
NIH/3T3-Rf-4-1[c]	10	4.2, 5.5
NIH/3T3-Rf-4-2[c,d]	5	1.1, 0.9
NIH/3T3	1	—[e], 0.3

a) Determined by densitometric scanning of Northern blot analysis.
b) Total intesity of all the β-actin spots; two different experiments were analyzed.
c) NIH/3T3 cells transformed by pEJ*ras*.
d) The spot of H-*ras* was faintly detected on the autoradiogram.
e) Intensity level was below the detection level of densitometry.

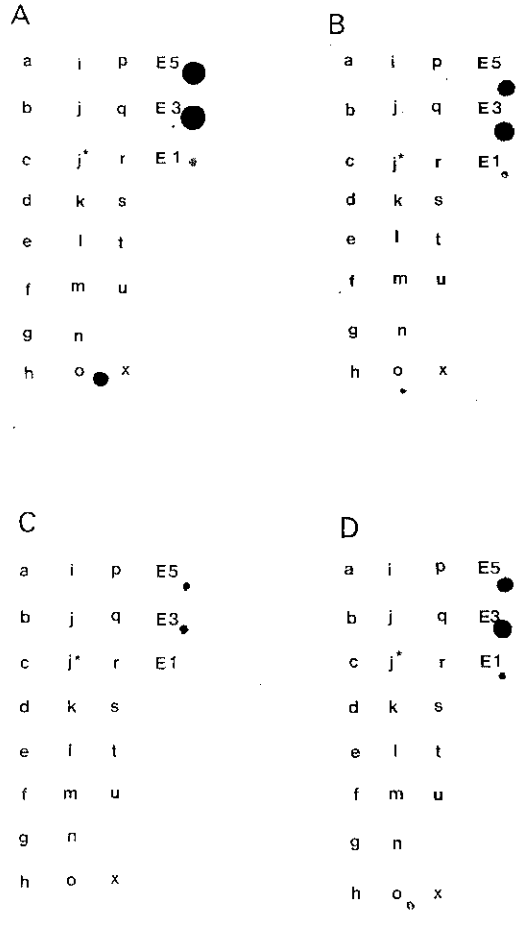

Fig. 2. Simultaneous screening of HL60 cells with cDNA probes synthesized under the conditions given in Table I. A, condition A; B, condition B; C, condition C; D, condition D. Letters in the autoradiograms indicate cloned oncogene DNAs: a, v-*src*; b, v-*yes*; c, v-*fgr* (γ-actin homologous sequence deleted); d, v-*abl*; e, v-*fps*; f, v-*fes* (cellular homolog is identical with v-*fps*); g, v-*ros*; h, v-*erb*B; i, v-*fms*; j, j*, v-*raf* (j is subcloned from j* as a v-*raf* specific sequence); k, v-*mos*; l, v-H-*ras*; m, v-K-*ras*; n, human N-*ras*; o, v-*myc*; p, v-*fos*; q, v-*myb*; r, v-*rel*; s, v-*erb*A; t, v-*sis*; u, human N-*myc*; x, pBR322; E1, human β-actin exon I; E2, human β-actin exons II and III; E3, human β-actin exon III; E4, human β-actin exons IV and V; E5, human β-actin exon V. Hybridization was performed with a variety of specific activities (A, 2×10^7 cpm/ml; B, 0.5×10^7 cpm/ml; C, 2.5×10^9 cpm/ml; D, 0.3×10^7 cpm/ml).

length of the cDNA probes was shorter (Fig. 1).

Purification of poly(A)$^+$ RNA from the same amount of total RNA under conditions A, C and D did not result in increased yield, specific activity or cDNA probe length (Table I, condition B; Fig. 1, lane B). This could be due to the degradation or loss of poly(A)$^+$ RNA during the purification process.[9]

We were able to detect c-*myc* overexpression using cDNA probes which were synthesized under the three conditions shown in Table I (Fig. 2, A, B, D). The spot o (*myc*) was detected most clearly when the cDNA probes synthesized under condition A were hybridized (Fig. 2, A). In contrast, the spot E1 (β-actin exon I) was not detected when the cDNA probes synthesized under condition C were hybridized (Fig. 2, C). This was because the synthesis of cDNA probes using condition C was not as effective as synthesis using other conditions (Fig. 1, lane C). As a result, we used condition A for the following experiments and the 5′-site of β-actin DNA as an indicator of cDNA probe synthesis.

Table III. Simultaneous Screening of Cell Lines and Primary Tumors

Cell line and tumor	Detected oncogene	Relative intensity to β-actin[a] (%)	Relative levels[b] to control[c]
Cell line			
Colorectal cancer			
HT 29	*myc*	0.6[d]	2–4
CCK-81	—	—	—
Thyroid cancer			
TCO-1	—	—	—
TCO-3	*myc*	0.8[d]	2–4
TCO-4	—	—	—
TCO-5	*myc*	0.9[d]	2–4
SENTO	—	—	—
Other human tumor			
GOTO (neuroblastoma)	N-*myc*	29	64–128
	N-*ras*	1.1	2–4
A1235 (astrocytoma)	*raf*	2.4	4–8
Non-human tumor			
MSBI (Marek's disease)	*myc*	2.9	8–16
	myb	4.8	64–128
Primary tumor			
Colorectal cancer			
CC1	*myc*	1.5	4–8
CC2	*myc*	2.1	4–8
CC3	—	—	—
CC4	—	—	—
CC5	*myc*	1.7	4–8
CC6	—	—	—
Other human tumor			
MEN-1 (thyroid cancer)	*myc*	0.6[d]	2–4
MK-1 (gastric cancer)	—	—	—
MFH-1 (malignant fibrohystiocytoma)	*myc*	1.4	4–8
LIP-1 (liposarcoma)	—	—	—

—: Not detected.

a) Total intensity of all the β-actin spots.

b) Determined with RNA dot blotting by a serial dilution procedure.

c) Human fibroblasts were used as a control for all human malignancies, except primary colorectal cancer and gastric cancer, and chick embryo fibroblasts for MSBI.

d) Faintly detected, but other spots were not detected.

Sensitivity of the Simultaneous Screening Method To estimate the detection limits of this method, we analyzed NIH/3T3 cells and three NIH/3T3 transformants using pEJ-*ras*[13] — with different levels of H-*ras* expression (Table II) — as a source of template RNA.

On the autoradiograms, the spot of H-*ras* was clearly detected in two transformants and faintly detected in another, but was not detected in NIH/3T3 cells (data not shown). The spots of the other oncogenes and pBR322 plasmid were barely detected. We also performed densitometric scanning of these autoradiograms to confirm whether the intensity of these spots was proportional to the population of probes.[14] A positive correlation was obtained between H-*ras* expression and the relative intensity of the H-*ras* spot compared to the total intensity of all β-actin spots. The intensity of the faintly detected spot was approximately 1% of the total intensity of all β-actin spots (Table II). The intensity of the other spots was less than 0.3% of the total intensity of all β-actin spots. These results suggest that a five-fold level of expression of H-*ras* is detectable on autoradiograms. This level of detection is equivalent to approximately 1% of the expression level of the β-actin gene by densitometric scanning.

It has also been estimated that this level is only 0.01–0.02% of the total population of cDNA probes, because β-actin mRNA is 1.0–2.0% of the total poly(A)$^+$ RNA.[15] This population represents a five- to ten-fold level of rare mRNA, including c-*myc* and c-H-*ras*, at 2–4 copies per normal cell.[16, 17] The level of other oncogene mRNAs is estimated to be equivalent to or several fold lower than that of c-*myc* and c-H-*ras*.[17, 18] Therefore, we concluded that the lower limit of detection for this method was about 10–20 copies per cell and this was sufficient to detect overexpression of almost all oncogenes in the cells examined.

Simultaneous Screening of a Variety of Cell Lines and Primary Tumors To evaluate whether oncogenes other than *ras* and *myc*, could be detected with this method, further analyses were performed on A431 cells,[19] NIH/3T3 cells transformed by v-*fgr* (unpublished work), and TPA (12-tetradecanoylphorbol-13-acetate)-stimulated Balb/3T3 cells,[20] in which overexpressions of *erb*B, *fgr*, and *fos* were reported, respectively. These sequences were clearly detected without cross-hybridization to the homologous sequences, i.e., tyrosine kinase family oncogenes for *erb*B and *fgr* (data not shown). Therefore, we assumed that our method was sufficiently effec-

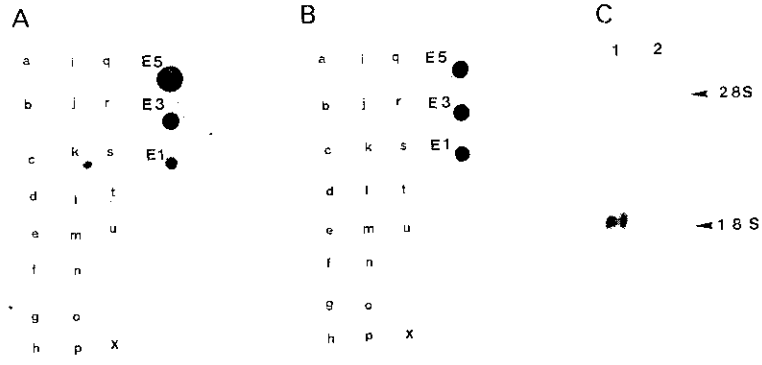

Fig. 3. Overexpression of *mos* in Syrian hamster cells transformed by X-ray-induced mouse osteosarcoma DNA. A: Simultaneous screening of transformed cells. B: Simultaneous screening of Syrian hamster cells. See the legend to Fig. 2 for spot identification. Hybridization and washing were performed under the same conditions. The specific activities of both probes were equivalent (2.0×10^7 cpm/ml). Exposure time was 24 hr. C: Northern blot analysis of the transformant (lane 1) and Syrian hamster cells (lane 2). Twenty micrograms of total RNA was blotted and hybridized with v-*mos* probe.

tive to detect specifically oncogene overexpression in the examined cells.

To confirm the utility of the simultaneous screening method, we screened 10 tumor cell lines and 10 primary tumors in which oncogene overexpression had not previously been determined. We subsequently detected elevated levels of five oncogenes (*myc*, N-*myc*, N-*ras*, *raf*, *myb*) in six tumor cell lines and five primary tumors (Table III). These results were further confirmed by Northern and dot blotting methods (data not shown). In this series of screenings we did not detect overexpression of oncogenes in the normal cells used as negative controls.

On the assumption that increased mRNA levels may be involved in altered gene expression, we examined two SHOK cells transformed by X-ray-induced mouse osteosarcoma DNA and human thyroid cancer DNA, respectively. The overexpression of *mos* was detected in the former transformant (Fig. 3), which suggests the involvement of altered *mos* gene expression. We also detected the additional and amplified *mos* sequence by Southern blot analysis.[21] In SHOK cells transformed by human thyroid cancer DNA, however, overexpression of the 20 oncogenes investigated was not found. Further analysis of this transformant showed the involvement of a new oncogene (unpublished work).

These results strongly suggest that the simultaneous screening method is useful in detecting overexpression of several known oncogenes simultaneously. It should also be noted that this method is superior to hybridization using prevailing RNA blotting methods, as it is less complicated and less time-consuming. Therefore, we conclude that the simultaneous screening method could be one of the most useful tools currently available for studies aimed at understanding malignancy and the process of carcinogenesis at the molecular level.

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