Analytical Biochemistry **295,** 17–21 (2001)
doi:10.1006/abio.2001.5171, available online at http://www.idealibrary.com on IDEAL®

Ribosomal 18S RNA Prevails over Glyceraldehyde-3-Phosphate Dehydrogenase and β-Actin Genes as Internal Standard for Quantitative Comparison of mRNA Levels in Invasive and Noninvasive Human Melanoma Cell Subpopulations

Didier Goidin,[1] Audrey Mamessier, Marie-Jeanne Staquet, Daniel Schmitt, and Odile Berthier-Vergnes[2]
INSERM U 346, affiliée CNRS, Edouard Herriot Hospital, F-69437 Lyon, France

Received December 27, 2000; published online June 13, 2001

The comparison of the gene expression profiles between two subpopulations of melanoma cells (1C8 and T1C3) derived from the tumor of one patient by cDNA array revealed differences in GAPDH and β-actin gene levels. These two housekeeping genes were up-regulated in invasive T1C3 melanoma cells compared to noninvasive 1C8 cells. Since cDNA array results were not confirmed by conventional RT-PCR throughout the exponential phase of amplification, we performed duplex relative RT-PCR using ribosomal 18S RNA as internal standard including competimer technology. Statistical analyses provided significant evidence that invasive T1C3 melanoma cells exhibited a twofold higher mRNA level of both GAPDH and β-actin than noninvasive 1C8 cells. This study demonstrates that the duplex relative RT-PCR procedure including ribosomal 18S RNA as internal standard and competimer technology is precise for RNA quantification and is tailored for cDNA array validation. Our data provide molecular evidence that cellular subpopulations of the same pathological origin are highly heterogeneous and extend the concept that the selection of an appropriate internal control for comparative mRNA analysis should be adapted to each model of human cancers.

Key Words: **ribosomal 18S RNA; GAPDH; β-actin; duplex relative RT-PCR; cDNA array; melanoma cells; invasive and metastatic potential.**

Metastatic progression is dependent upon an accumulation of multiple genetic and epigenetic alterations in malignant cells (1, 2). The identification of genes specifically overexpressed or repressed in metastatic cancer cells represents a key step toward the understanding of the malignant transformation process (3). Therefore, comparative analyses of gene expression profiles in a variety of cancer cell lines with different metastatic potential have been the subject of intense research (4–7).

Semiquantitative analysis of gene expression level by Northern blots, dot blots, ribonuclease protection assays, reverse transcription–polymerase chain reaction (RT-PCR)[3] and more recently cDNA array needs standardization. Housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are widely used as internal standards. Because these two proteins are essential for the maintenance of cell function, it is generally assumed that they are constitutively expressed at similar levels in all cell types and tissues. However, several reports indicate that the expression of these housekeeping genes varies across tissues (8) and cell types (9), during cell proliferation (10) and development (11). Additionally, GAPDH mRNA level is widely altered in cultured cells in response to various stimuli, including hypoxia (12), insulin (13), dexamethasone (11), mitogens (14), and epidermal growth factor (EGF) (15) as well as in virally transformed or oncogene-transfected fibroblasts (15).

[1] A "Scientifique du Contingent" from DSP/DGA/STTC (Ministry of Defense).

[2] To whom correspondence should be addressed at INSERM U 346, Hôpital Edouard Herriot, Pavillon R, 69437 Lyon 03, France. Fax: + 33(0)4 72 11 02 90. E-mail: berthier@lyon151.inserm.fr.

[3] Abbreviations used: RT-PCR, reverse transcription–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGF, epidermal growth factor.

0003-2697/01 $35.00

Analyses of gene expression in a series of cell lines derived from the same pathological provenance is often the first step toward gaining an insight into the genetic alterations of a particular cancer. The emerging technology of cDNA array provides a powerful tool to rapidly identify genes whose expression is altered in human cancers (7, 16). Obtaining valid, quantitative results from such studies requires verification by standard techniques such as Northern blotting, ribonuclease protection assays, or RT-PCR. However, confirmation of array data is sometimes unreliable. This may be due to the fact that quantification of gene levels is based upon the use of common housekeeping genes whose expression is likely to be modified in tissues and cell types (8, 9). In the present study, we compared the level of expression of two common housekeeping genes, GAPDH and β-actin, in subpopulations of human cancer cells differing in their invasive and metastatic abilities (17–19). Unlike studies which compare cell lines derived from different patients (4–6), here we used two subpopulations of melanoma cells (1C8 and T1C3 clones) selected from the tumor of one patient (17), allowing comparison between genetically similar cell populations. cDNA array demonstrated that invasive T1C3 melanoma cells exhibited a twofold higher mRNA level for both GAPDH and β-actin than noninvasive 1C8 cells. These results are confirmed by a duplex relative RT-PCR using ribosomal 18S rRNA as internal standard including competimer technology but not by conventional RT-PCR.

MATERIALS AND METHODS

Human Melanoma Cells and Culture

The selection procedure of IC8 and T1C3 human melanoma cell clones, derived from a single parental cell line, have been previously described. Only T1C3 cells promote metastases in animals (17, 18) and penetrate through the dermal–epidermal junction of human skin reconstructs (19). Melanoma cells were cultured for 3 days as monolayers in McCoy's 5A medium supplemented with 10% fetal calf serum (Life Technologies, Cergy-Pontoise, France). The cultures, maintained at 37°C and 5% CO_2 in a humidified atmosphere, were regularly checked for the absence of mycoplasma.

cDNA Array Analysis

Gene expression of GAPDH and β-actin was analyzed using the human cytokine expression array (R&D Systems, Abingdon, UK), according to the manufacturer's recommendations. Briefly, 2 μg of total RNAs prepared with SV total RNA isolation system including DNase treatment (Promega, Charbonnières, France) was reverse transcribed using human cytokine-specific primers (R&D Systems) in the presence of [^{33}P]dCTP (10 mCi/ml; 3000 Ci/mmol; DuPont NEN Research Products, Boston, MA). After Sephadex G-25 spin column purification (R&D Systems), labeled cDNA probes (10^6 cpm/ml) were applied to the membranes overnight at 65°C. After extensive washes, hybridized membranes were exposed to a phosphor screen for 18 h and scanned using a personal molecular PhosphorImager Fx system (Bio-Rad SA, Ivry Sur Seine Cedex, France) at the Institut de Recherche Pierre Fabre, St Julien en Genevois, France. The intensity of the hybridization signal for each gene was quantified using Quantity 1 software (version 1.1, Bio-Rad) and normalized to the sum of intensity of all genes present on the membrane.

RT-PCR Analysis

One microgram of total RNA was reverse-transcribed with random primers using the reverse transcription system (Promega), following manufacturer's instructions. PCR was performed in a final volume of 50 μl containing 100 ng cDNA of template, 0.4 μM of each primer (Genset, Evry, France, see below), 0.2 mM of each dNTPs, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Promega). Fifteen to 29 cycles of PCR were carried out with 30-s denaturation at 94°C, 30-s annealing at 62°C followed by 30-s extension at 72°C. Cycling was started by 5-min denaturation at 94°C and terminated by 10-min incubation at 72°C. All PCR experiments were performed using thermocycler (Perkin–Elmer, Paris, France). The primers used recognized GAPDH gene sequence (forward primer, ACCACAGTCCATGCCATCAC; reverse primer, TCCACCACCCTGTTGCTGTA; 452 bp) and β-actin gene sequence (forward primer, GGCGACGAGGCCCAGA; reverse primer, CGATTTCCCGCTC GGC; 463 bp). Amplicons were visualized on 2% agarose gels containing SYBR Green (FMC/Tebu, Le Perray en Yvelines, France). The intensity of each band was determined by densitometric analysis of gels using Kodak DC120 zoom digital camera and Kodak 1D image analysis software (Gibco BRL, Cergy Pontoise, France).

Duplex Relative RT-PCR Analysis

GAPDH and β-actin were coamplified with ribosomal 18S RNA (amplicon: 324 bp) for 20 cycles, under conditions described above. For both, GAPDH and β-actin, duplex PCR were proceeded at two different ratio of 18S primers/competimer: 1/9 and 2/8 as proposed by the manufacturer. To reduce 18S RNA amplification signal, we used the Quantum RNA alternate 18S RNA internal standard (Ambion, Clinisciences, Montrouge, France) including Ambion's competimer

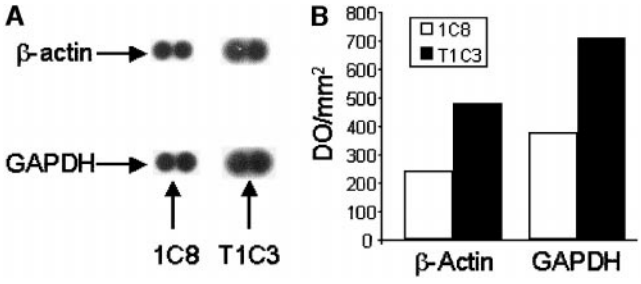

FIG. 1. Comparison of gene expression levels of GAPDH and β-actin in melanoma cells from invasive T1C3 and noninvasive 1C8 clones, by cDNA expression array. Nylon membrane containing duplicate cDNAs fragments was hybridized with 1C8 or T1C3 cDNA probes (see Materials and Methods). (A) Membrane images displayed by phosphoimager. (B) Relative intensity for each gene was measured using Image Quant software and normalized to the sum of all genes detected on the membrane.

technology which allowed modulation of the amplification efficiency of a PCR template without affecting the performance of others targets in a duplex or multiplex PCR. The 18S RNA competimers are identical to 18S RNA primers, but are modified at their 3′ end to block extension of DNA polymerase. Mixing 18S RNA primers with increasing amounts of 18S RNA competimer allowed a decrease of amplification efficiency of 18S RNA template without loss of relative quantitation. Results were expressed as the mean of the ratio between the optical density (OD) of GAPDH or β-actin amplicons to OD of 18S amplicon ± SD ($n = 4$). The significance of the differences in GAPDH and β-actin mRNA levels between the two melanoma clones was determined by one-way ANOVA using Stat View software (Alysyd Corp., Meylan, France) including Fisher's protected least significant difference test, Scheffe's test, and Bonferroni/Dunn's test.

RESULTS AND DISCUSSION

The gene expression level of GAPDH and β-actin was compared in two subpopulations of human melanoma cells (1C8 and T1C3 clones) derived from the tumour of one patient (17, 18). Only T1C3 cells produce spontaneous metastatic nodules in the lungs of rats in a fashion analogous to the evolution of melanoma in humans (17, 18) and are able to penetrate through an authentic dermal–epidermal junction in human skin reconstructs (19). Using human cytokine cDNA expression array, the hybridization signal of each gene, represented by two parallel dots, revealed differences between the two clones (Fig. 1A). Compared with noninvasive 1C8 melanoma cells, invasive T1C3 melanoma cells exhibited a twofold higher intensity value for both GAPDH and β-actin dots (Fig. 1B).

To validate the cDNA array results, GAPDH and β-actin gene expressions were analyzed by conventional RT-PCR. As shown in Fig. 2, the respective amplification pattern of GAPDH and β-actin genes differed between the two clones from 17 to 25 cycles. Under these conditions, PCR products were accumulating at a linear rate with the highest level in T1C3 cells compared to 1C8 cells. These findings reflect differences in the original abundance of both genes between the two clones. In contrast, the amplification pattern was similar for both clones as the number of PCR cycles increased (above 25) (Fig. 2), showing that conventional RT-PCR may mask significant variations and especially those detectable with cDNA array. This observation emphasizes the fact that, under these conditions, RT-PCR should only be considered as a qualitative tool.

To evaluate mRNA levels quantitatively, a duplex relative RT-PCR using ribosomal 18S RNA (18S rRNA) as internal standard was carried out, since as far as we know, variation in ribosomal RNA expression has never been reported (8, 20). Indeed, melanoma cells from both clones expressed similar levels of 18S rRNA, as visualized by ethidium bromide staining of denaturing agarose gel (unpublished data). We used competimers which are identical in sequence to 18S rRNA primers but are chemically modified such that they cannot

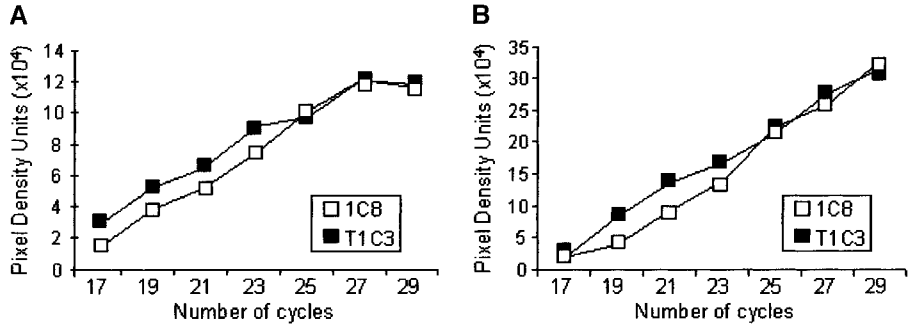

FIG. 2. PCR analysis of GAPDH and β-actin in melanoma cells from invasive (T1C3) and noninvasive (IC8) clones. β-Actin (A) and GAPDH (B) were amplified from 17 to 29 cycles. Stained band intensity was determined by densitometric analysis of using Kodak DC120 zoom digital camera and Kodak 1D image analysis software, after visualization on 2% agarose gels containing SYBR Green.

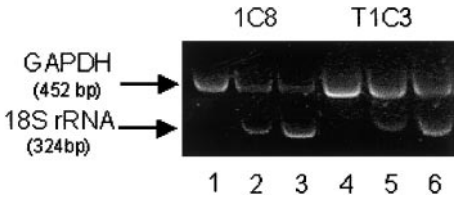

FIG. 3. Duplex relative RT-PCR analysis of GAPDH with 18S rRNA as internal control from noninvasive melanoma cells (clone IC8; lanes 1–3) and invasive melanoma cells (clone T1C3; lanes 4–6). Duplex relative RT-PCR with 18S rRNA as an internal control was performed using GAPDH primers alone (lanes 1 and 4); primers for GAPDH and 18S rRNA with competimer at two different ratios of primers/competimer: 1/9 (lanes 2 and 5); 2/8 (lanes 3 and 6). This is a representative experiment of three.

be extended during PCR and are resistant to nuclease degradation. Figure 3 illustrates the expression level of GAPDH in both melanoma clones using a duplex relative RT-PCR assay. By using a 18S rRNA primers/competimer ratio of 1/9, coamplification with 18S rRNA vs GAPDH alone did not modify GAPDH amplicon signal in T1C3. In contrast, coamplification of GAPDH with 18S RNA decreased GAPDH amplicon signal in 1C8 cells (Fig. 3). Coamplification of β-actin with 18S rRNA gave similar results (data not shown). We next quantified the gene expression level of GAPDH and β-actin in the two clones using 18S rRNA as internal control. Amplicons were quantified by densitometric analysis and the ratio between OD of GAPDH or β-actin amplicons to OD of 18S RNA was calculated according to the quantity of competimer used (18S RNA primers/competimer ratio: 1/9, 2/8) (Table 1). After statistical analysis, the ratio T1C3/1C8 for each quantity of competimer was computed. Melanoma cells from the invasive T1C3 clone displayed a twofold increased expression level of both genes compared to melanoma cells from the noninvasive 1C8 clone. This finding correctly reflects the differences observed by cDNA array procedure.

To our knowledge, this is the first report demonstrating that subpopulations of human malignant cells selected from the same tumor of one patient, which means with the same genetic background, strongly differed in the expression level of GAPDH and β-actin housekeeping genes widely used as RNA standards. More importantly, invasive and metastatic melanoma cells exhibited a twofold higher level of both GAPDH and β-actin than noninvasive and nonmetastatic melanoma cells. Based upon these observations, we conclude that GAPDH and β-actin are not suitable as RNA-loading controls for RT-PCR to validate a comparison of expression levels of specific mRNA between subpopulations of human cells selected from the same tumor. To overcome this problem, 18S rRNA is proposed as a reliable standard for the use of relative RT-PCR in gene expression, as also pointed out for rat and mouse tissues (25). Indeed, the level of ribosomal RNAs, which constitute up to 80% of the total RNA, is thought to be less likely to vary under conditions that affect the expression of mRNAs, and moreover, they are transcribed by a distinct RNA polymerase.

A high level of GAPDH gene correlates with the aggressiveness of both breast cancer (21) and renal cell carcinoma (22). Moreover, GAPDH mRNA is found to be elevated in lung and colon cancer tissues (23), in pancreatic adenocarcinoma cell lines (24) when compared to their respective normal counterparts. Our data contribute additional evidence that a high level of GAPDH gene expression may be characteristic of human cancers, and point to abundance in the invasive subpopulations preexisting in the primary tumor.

Identification of genes differentially expressed in selected subpopulations of cancer cells (normal vs tumoral; metastatic vs nonmetastatic) by cDNA array needs to be quantitatively verified. This study demonstrates the power of duplex relative RT-PCR including ribosomal 18S RNA for the verification of differential gene expression in cellular subpopulations of human cancers instead of conventional RT-PCR using housekeeping genes. Moreover, our data provide molecular evidence that cellular subpopulations of the same pathological origin are highly heterogeneous and ex-

TABLE 1

Quantitation of GAPDH and β-Actin mRNA Levels in Human Melanoma Cells from IC8 and T1C3 Clone

	Ratio 18S/competimer	1C8 (**3**)	T1C3 (**4**)	*P*	T1C3/1C8 (**5**)
GADPH (**1**)	1/9	0.103 ± 0.029	0.243 ± 0.080	0.0466*	2.36
	2/8	0.070 ± 0.025	0.140 ± 0.008	0.0351*	2.00
β-Actin (**2**)	1/9	1.410 ± 0.164	3.057 ± 0.136	0.0002**	2.17
	2/8	0.390 ± 0.072	0.793 ± 0.150	0.0138*	2.03

Note. Coamplification of GAPDH (**1**) or β-actin (**2**) with 18S rRNA in two differents ratios of 18S primer/competimer (1/9 and 2/8). The mRNA level of GAPDH and β-actin in both clones (**3** and **4**) was normalized to the 18S rRNA control according to the following formula: OD GAPDH or β-actin/OD 18S rRNA. For each gene, results are expressed as the mean ± SD (n = 4). The ratio T1C3/1C8 (**5**) corresponds to the mean value of T1C3 (**4**)/the mean value of 1C8 (**3**).

* Statistical analysis was carried out by one-way ANOVA (*P*).

tend the concept that the selection of an appropriate internal control for quantitative RNA analysis should be adapted to each model of human cancers.

ACKNOWLEDGMENTS

We thank Dr. J. F. Gauchat (Institut de Recherche Pierre Fabre, St Julien en Genevois, France) for his help in cDNA array analysis and Martine Gaucherand for her technical assistance. The authors are grateful to Drs. J. F. Gauchat and T. Ouimet (INSERM U109, Paris) for their critical reading of the manuscript. This work was supported by the Institut National de la santé et de la Recherche Médicale (INSERM) and in part by specific grants from the Comité Départemental du Rhône de la Ligue Nationale contre le Cancer.

REFERENCES

1. Nowell, P. C. (1986) Mechanisms of tumor progression. *Cancer Res.* **46,** 2203–2207.
2. Weinberg, R. A. (1989) Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res.* **49,** 3713–3721.
3. Nicolson, G. L. (1987) Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. *Cancer Res.* **47,** 1473–1487.
4. Xin, H., Stephans, J. C., Duan, X., Harrowe, G., Kim, E., Grieshammer, U., Kingsley, C., and Giese, K. (2000) Identification of a novel aspartic-like protease differentially expressed in human breast cancer cell lines. *Biochim. Biophys. Acta* **1501,** 125–137.
5. Yu, L., Hui-chen, F., Chen, Y., Zou, R., Yan, S., Chun-xiang, L., Wu-ru, W., and Li, P. (1999) Differential expression of RAB5A in human lung adenocarcinoma cells with different metastasis potential. *Clin. Exp. Metastasis* **17,** 213–219.
6. Zendman, A. J., Cornelissen, I. M., Weidle, U. H., Ruiter, D. J., and van Muijen, G. N. (1999) TM7XN1, a novel human EGF-TM7-like cDNA, detected with mRNA differential display using human melanoma cell lines with different metastatic potential. *FEBS Lett.* **446,** 292–298.
7. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., Sampas, N., Dougherty, E., Wang, E., Marincola, F., Gooden, C., Lueders, J., Glatfelter, A., Pollock, P., Carpten, J., Gillanders, E., Leja, D., Dietrich, K., Beaudry, C., Berens, M., Alberts, D., and Sondak, V. (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* **406,** 536–540.
8. de Leeuw, W. J., Slagboom, P. E., and Vijg, J. (1989) Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control. *Nucleic Acids Res.* **17,** 10137–10138.
9. Spanakis, E. (1993) Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. *Nucleic Acids Res.* **21,** 3809–3819.
10. Mansur, N. R., Meyer-Siegler, K., Wurzer, J. C., and Sirover, M. A. (1993) Cell cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in normal human cells. *Nucleic Acids Res.* **21,** 993–998.
11. Oikarinen, A., Makela, J., Vuorio, T., and Vuorio, E. (1991) Comparison on collagen gene expression in the developing chick embryo tendon and heart. Tissue and development time-dependent action of dexamethasone. *Biochim. Biophys. Acta* **1089,** 40–46.
12. Zhong, H., and Simons, J. W. (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem. Biophys. Res. Commun.* **259,** 523–526, doi:10.1006/bbrc.1999.0815.
13. Alexander, M., Curtis, G., Avruch, J., and Goodman, H. M. (1985) Insulin regulation of protein biosynthesis in differentiated 3T3 adipocytes. Regulation of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **260,** 11978–11985.
14. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1990) Differential regulation of Na+/H+ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J. Biol. Chem.* **265,** 19393–19396.
15. Matrisian, L. M., Glaichenhaus, N., Gesnel, M. C., and Breathnach, R. (1985) Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. *EMBO J.* **4,** 1435–1440.
16. Goidin, D., Kappeler, L., Perrot, J., Epelbaum, J., and Gourdji, D. (2000) Differential pituitary gene expression profiles associated to aging and spontaneous tumors as revealed by rat cDNA expression array. *Endocrinology* **141,** 4805–4808.
17. Zebda, N., Bailly, M., Brown, S., Dore, J. F., and Berthier-Vergnes, O. (1994) Expression of PNA-binding sites on specific glycoproteins by human melanoma cells is associated with a high metastatic potential. *J. Cell. Biochem.* **54,** 161–173.
18. Berthier-Vergnes, O., Zebda, N., Bailly, M., Bailly, C., Dore, J. F., Thomas, L., and Cochran, A. J. (1993) Expression of peanut agglutinin-binding glycoconjugates in primary melanomas with high risk of metastases. *Lancet* **341,** 1292.
19. Bechetoille, N., Haftek, M., Staquet, M. J., Cochran, A. J., Schmitt, D., and Berthier-Vergnes, O. (2000) Penetration of human metastatic melanoma cells through an authentic dermal–epidermal junction is associated with dissolution of native collagen types IV and VII. *Melanoma Res.* **10,** 427–434.
20. Bhatia, P., Taylor, W. R., Greenberg, A. H., and Wright, J. A. (1994) Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for Northern blot analysis of cell lines of varying malignant potential. *Anal. Biochem.* **216,** 223–226, doi:10.1006/abio.1994.1028.
21. Revillion, F., Pawlowski, V., Hornez, L., and Peyrat, J. P. (2000) Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. *Eur. J. Cancer* **36,** 1038–1042.
22. Vila, M. R., Nicolas, A., Morote, J., de, I., and Meseguer, A. (2000) Increased glyceraldehyde-3-phosphate dehydrogenase expression in renal cell carcinoma identified by RNA-based, arbitrarily primed polymerase chain reaction. *Cancer* **89,** 152–164.
23. Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K., and Sakiyama, S. (1987) Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res.* **47,** 5616–5619.
24. Schek, N., Hall, B. L., and Finn, O. J. (1988) Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human pancreatic adenocarcinoma. *Cancer Res.* **48,** 6354–6359.
25. Flouriot, G., Nestor, P., Kenealy, M. R., Pope, C., and Gannon, F. (1996) An S1 nuclease mapping method for detection of low abundance transcripts. *Anal. Biochem.* **237,** 159–161, doi:10.1006/abio.1996.0219.