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Communication

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Identification of Novel Genes Differentially Expressed in PMA-induced HL-60 Cells Using cDNA Microarrays

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Identification of normal growth and differentiation-inducing proteins and their interaction in normal development have made it possible to elucidate the molecular basis of normal development and the mechanisms uncoupling growth and differentiation during tumor development. The development of cancer and the experimental reversal of tumorigenicity are accompanied by complex changes in patterns of gene expression. cDNA microarrays provide a powerful tool for studying these phenomena. In the present study, a high-density microarray of human cDNA elements was used to search for differences in gene expression associated with differentiation of human promyelic leukemia HL-60 cells. Microarrays containing 3,063 human cDNAs were printed on glass slides with high-speed robotics. These DNA 'chips' were used to quantitatively monitor differential expression of the cognate human genes using a highly sensitive two-color hybridization assay. The identification of known and novel phorbol ester-regulated genes in hematopoietic progenitor cells demonstrates the sensitivity of the assay.

Keywords: cDNA Microarray; Differentiation; HL-60.

Introduction

Hematopoiesis gives rise to blood cells of different lineages throughout normal life. Abnormalities in this developmental program lead to blood cell diseases, including leukemia. The establishment of a cell culture system for the clonal development of hematopoietic cells made it possible to discover proteins that regulate cell viability, multiplication and differentiation of different hematopoietic cell lineages, and the molecular basis of normal and abnormal blood cell development. Malignancy can be suppressed in certain types of leukemic cells by inducing differentiation with cytokines that regulate differentiation pathways. This created the basis for the clinical use of normal hematopoiesis or with other compounds that use alternative 'differentiation therapy' (Sachs, 1993). Identification of normal growth and differentiation-inducing proteins and how they interact in normal development has made it possible to identify the molecular basis of normal development and the mechanisms that uncouple growth and differentiation so as to produce tumor cells. The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, can be induced to differentiate *in vitro* to a number of different cell types. Studies with this leukemic cell line have shown invaluable outcomes in a variety of different areas. These include: (A) providing insight into the differentiation; (B) suggesting new therapeutic approaches for patients with leukemia; (C) providing a ready source of cDNA for cloning of important granulocyte and monocyte enzymes and monokines; (D) providing insight into a possible mechanism of rapid regulation of gene transcription; and (E) serving as an invaluable model for studying specific cellular oncogene expression in relation to particular hematopoietic differentiative lineages (Collins, 1987).

Cells of the HL-60 line were induced to differentiate into granulocytes with DMSO or retinoic acid (Collins, 1978) or into monocyte/macrophage-like cells with PMA or vitamin D3 (McCarthy *et al.*, 1983; Rovera *et al.*, 1979).

HL-60 cells harbor specific receptors for PMA (Cooper *et al.*, 1982), and considerable evidence for HL-60 shows that the PMA receptor is protein kinase C (Vandenbark *et al.*, 1984). PMA is a plant derivative that appears to mimic the effect of naturally occurring compounds, such as endogenous diacylglycerol, resulting in prolonged activation of protein kinase C. This

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Abbreviations: DMSO, dimethylsulfoxide; PMA, phorbol 12-myristate 13-acetate.

activation of protein kinase C in HL-60 cells could result in the phosphorylation of specific substrate proteins, leading to the macrophage-like phenotypic changes induced by PMA (Collins, 1987).

In order to identify other genes which are differentiation-related in HL-60, a human cDNA microarray was used. Recently, cDNA microarray technologies have been developed and used to identify differentially expressed genes. This technique has made it easy to monitor thousands of genes simultaneously for differential expression between two different samples (Schena *et al.*, 1995). In the present study, we have identified many differentially expressed genes in the differentiated HL-60 cell line, including many novel genes using a cDNA microarray.

Materials and Methods

Materials The RPMI 1640 medium was provided by Gibco BRL (NY, USA). Fetal bovine serum was by Hyclone (Utah, USA). [α-^{32}P]dCTP was purchased from NEN (Boston, USA). PMA was from Sigma (St Louis, MO, USA). RNA wiz was purchased from Ambion (Texas, USA).

Cell cultures and macrophage differentiation HL-60 cells were provided by American Type Culture Collection and were cultured in 75-cm^2 tissue culture flasks. The cells were seeded at an initial concentration 5×10^5 cells/ml in an RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were cultured at 37°C in humidified air containing 5% CO_2. Macrophage differentiation was induced by addition of 160 nmol/l of PMA (Sigma) and differentiated adherent cells were harvested after 48 h (Manfredini *et al.*, 1997).

Human cDNA clone All the cDNA clones used in this study were obtained from Human Stromal Cells cDNA Bank maintained in Kyungpook National University, Daegu, Korea.

Preparation of Microarrays

Preparation of DNA samples cDNA microarrays were produced by spotting PCR products representing specific genes onto a glass slide. Typically, the PCR products were purified by precipitation to remove unwanted salts, detergents, and PCR primers. All insert cDNAs were amplified by PCR for 35 cycles with C6 amine-modifed T3 and T7 universal primers (IDT, USA). For each 96-well plate to be amplified (MJ Research, USA), PCR mixtures containing 1× PCR buffer, 0.2 μM each primer, 2.5 U Taq DNA polymerse (Promega), and 0.2 mM dNTPs (Amersham Pharmacia biotech) were prepared. Thermal cycling was done for 35 cycles at 94°C for 60 s, at 57°C for 90 s, and at 72°C for 120 s and an additional extension period at 72°C for 7 min. Three microliters of each PCR reaction was checked by electrophoresis in 1% agarose gel. The remaining PCR product was precipitated in the 96-well plates by addition of 3 M sodium acetate (pH 5.2) and pure ethanol. The precipitated the DNA was washed once with 75% ethanol and the DNA was dried in air. The DNA pellets were suspended in 3× SSC and transferred from the 96-well plates to 384-well plates (ABgene House, UK). Using a robotic spotting device (Cartesian PixSYS 5500), the DNA was printed onto amino-silane coated slide glasses (CMT-GAPS^{TM} Corning). A total of 3,063 cDNAs, representing 3,031 human clones and 32 controls, were arrayed in areas of 1.8 cm^2.

Postprocessing of arrays After printing, the slides were allowed to dry under vacuum for several days and were washed twice in 0.2% SDS for 2 min and twice in water at 95–100°C for 2 min to allow DNA denaturation. Then, the slides were allowed to dry thoroughly at room temperature (~5 min) and were rinsed in sodium borohydride solution (1.0 g of $NaBH_4$ dissolved in 300 ml of PBS and 100 ml of 100% ethanol) for 5 min at room temperature to reduce free aldehydes. The slides were washed three times in 0.2% SDS for 1 min each at room temperature and once in water for 1 min at room temperature. The slides were submerged in water at 95–100°C for 2 s, air-dried, and stored in the dark at 25°C.

Preparation of fluorescent DNA probe from mRNA The total RNA was extracted from the HL-60 cells using a modified acid phenol method. Briefly, the cells were harvested and lysed with RNA wiz (Ambion). The lysate was cleared and extracted with chloroform. The RNA was precipitated, resuspended in DEPC-treated water, and quantified using a UV spectrophotometer (DU530, Beckman). Ten micrograms of the total RNA was loaded onto denaturing 1.0% agarose formaldehyde gels. mRNA was isolated from the total RNA with an oligotex mRNA midi kit (Qiagen, Chatsworth, CA, USA). Cy3- and Cy5-dUTP (Amersham) fluorescent dye labelled probes were made by reverse transcription with Superscript II RNase H-reverse transcriptase (Gibco BRL). Each reverse transcription reaction contained 2.0–5.0 μg of mRNA. Following the reverse transcription step, samples were treated with 1.0 μl of 1.5 M sodium hydroxide for 10 min at 65°C, then neutralized by adding 468 μl of TE (pH 7.4). The probe volume was reduced using a Microcon30 (Millipore, USA), and Cy3 and Cy5 probes were mixed and added 1.0 μl of 5% SDS and 4.0 μl of 20× SSC (final concentration, 4× SSC) were added, and the total volume was made to 20 μl with TE buffer.

Microarray hybridization The arrays were prehybridized for 20 min in a solution containing BSA (1%) and were hybridized for 18 h at 50°C in a solution containing poly A blocker and yeast tRNA. The arrays were washed for 5 min at room temperature in low-stringency wash buffer (0.1× SSC/0.1% SSC), then for 5 min in high-stringency wash buffer (0.1× SSC).

Scanning and image analysis Fluorescence intensities at the immobilized targets were measured using Scanarray 4,000 with a laser confocal microscope (GSI Lumonics, USA). The two fluorescent images (Cy3 and Cy5) were scanned separately from a confocal microscope, and color images were formed by arbitrarily assigning differentiated cell intensity values into the red channel and a control intensity into the green channel and

the data were analyzed using Quantarray software (version 2.0.0.0a, GSI Lumonics). The results were also analyzed by normalization between the images to adjust for the different efficiencies in labeling and detection with the two different fluors. This was achieved by matching the detection sensitivities with a set of 32 internal control genes (GAPDH).

Northern blot analysis The total RNA was extracted from HL-60 cells using a modified acid phenol method. Briefly, cells were harvested into RNA wiz (Ambion). The lysate was cleared and extracted with chloroform. The RNA was precipitated, resuspended in DEPC-treated water, and quantified by A260/A280 measurement. 10 micrograms of the total RNA was loaded onto denaturing 1.0% agarose formaldehyde gels. Separated RNA was transferred to a nylon membrane and cross-linked with 240 mJ of UV irradiation in a Stratalinker (Stratagene). The membranes were prehybridized for 4 h and hybridized for 36 h at 42°C in a solution containing 50% formamide. Fragments of each cDNA were labeled with [α-^{32}P]dCTP by random hexamer priming using a Megaprime labelling kit (Amersham). Northern blots were washed at high stringency and exposed to X-ray film.

Results and Discussion

Gene discovery during macrophage differentiation Human cDNA microarray sets described to data represent a subset of the genes expressed in the human genome. They have provided valuable tools in analyzing the expression profiles of alveolar rhabdomyosarcoma (Khan *et al.*, 1998), fibroblasts in response to serum (Iyer *et al.*, 1999), hematopoetic cells in response to differentiation inducer (Tamayo *et al.*, 1999), acute promyeloic leukemia cells in response to retinoic acid (Rusiniak *et al.*, 2000), and of primary human fibroblasts by activation of c-MYC (Coller *et al.*, 2000). Cell lines, modeling the differentiation process have been extensively used over the past decade to study expression of dozens of individual genes. In this study, we tried to take advantage of a more global approach by cDNA microarray. Templates for the genes of interest were obtained and amplified by PCR. Following purification and quality control, aliquots ($\sim$1 nl) were printed on coated glass microscope slides using a computer-controlled robot. mRNAs from both test and reference samples were labeled with either Cy3- or Cy5-dUTP using a single round of reverse transcription. The fluorescent targets were pooled and allowed to hybridize under stringent conditions. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner (Scanarray 4,000) were imported into software (Quantarray) in which the images were pseudocolored and merged. The data from three independent hybridization experiments were viewed as a normalized ratio (that is, Cy5/Cy3) in which significant deviations from 1 (no change) were indicative of increased (>2.0) or decreased (<0.5) levels of gene expression relative to the reference sample. Hybridization of the Cy3-dUTP-labeled cDNA is represented as a green signal, and hybridization of the Cy5-dUTP-labeled cDNA is represented as a red signal (Fig. 1).

In the present study, we monitored gene expression with a cDNA microarray representing 3,063 cDNAs isolated from human stromal tissues. Among the total of 3,063 cDNA clones, 110 cDNA elements were upregulated (>2.0) and 113 elements were downregulated (<0.5) during induced differentiation of HL-60 cells. Several elements are presented in Table 1. Figure 2 shows the scatter plot of this experiment. To confirm the microarray results, the mRNA level for each of the genes was measured by Northern blot analysis. We chose five genes from Table 1 for Northern blot analysis. Northern blot analysis confirmed the microarray results indicating upregulation by PMA. Four of the genes that displayed PMA induction, including the three unknown genes, exhibited elevated mRNA levels (Fig. 3). The sequences of those unknown expressed sequence tags (EST) clones are listed in Fig. 4.

Among the differentially expressed genes in this experiment, a fair number of genes were already known

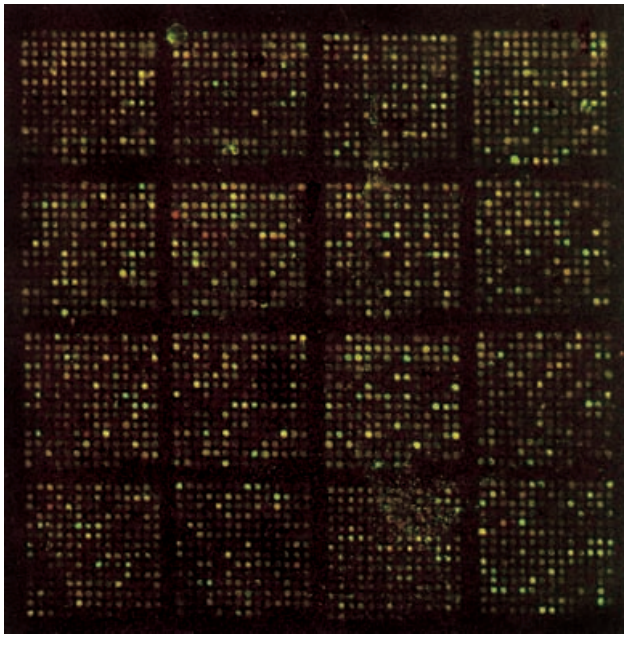

Fig. 1. The result of the microarray is shown for a hybridization comparing RNA isolated at 48 h after PMA treatment. The total microarray contains 3,063 genes, which were derived from human mesenchymal cell cDNA libraries. mRNA from non-treated cells was labeled with Cy3-deoxy uridine triphosphate (dUTP) (green fluorescence), and PMA treated cells were labeled with Cy5-dUTP (red fluorescence).

Table 1. Microarray elements corresponding to differentially expressed genes.

Identification	Clone ID	Cy5/Cy3 ratio	GeneBank no.
Increased			
Unknown	B1443	2.00	
Unknown	K1413	2.33	AL049929
Unknown	M1985	2.04	
Unknown	P1725	2.00	
CD9	M3380	2.19	NM_007657
Annexin A2 (ANXA2)	M4903	4.76	NM_00403
CAPL/mts1	K1136	2.29	M80563
CAPL/mts1	P0852	2.92	M80563
Cathepsin B	K0352	5.05	NM_001908
Galectin 1	M3639	9.34	NM_002305
MGC-24/CD164	K1201	5.70	D14043
MRJ gene for a member of the DNAJ family	M4325	6.78	NM_00549
sgk	K0192	5.82	U88666
sgk	B1027	7.29	U88666
TIMP 1	M0747	4.16	X03124
TIMP 1	M0389	4.57	X03124
p21/WAF1	P0251	2.47	U03106
Decreased			
Fibrillarin	B0915	0.40	M59849
HMG-2	M4371	0.29	X62534
PCNA	K1285	0.19	J04718

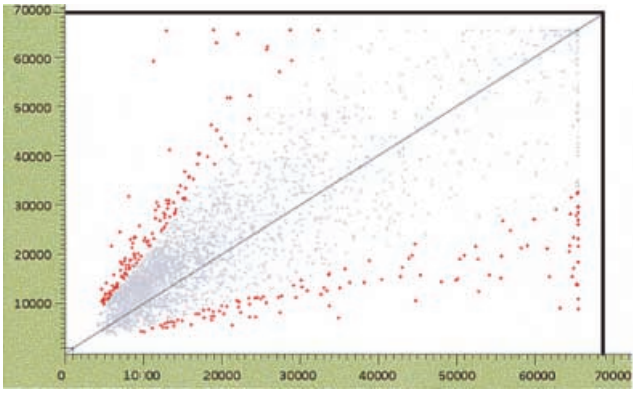

Fig. 2. Scatter plot of the microarray result. Those spots exhibiting a minimum level of expression of intensity of >1,000 fluorescence units (on a scale of 0-65,535 fluorescence units) were calculated. Each spot was plotted according to the fluorescence intensity of Cy3 (X-axis) and Cy5 (Y-axis). Those spots showing more than a two fold difference between the Cy3 and Cy5 signals were considered as differentially expressed genes by PMA induction. 110 cDNA elements were upregulated (>2.0) and 113 elements were downregulated (<0.5) during the induced differentiation of HL-60 cells.

as participants in differentiation-related. These are cathepsin B, CD 9, mts1/CAPL, galectin 1, sgk, MGC-24/CD164, and p21/WAF1.

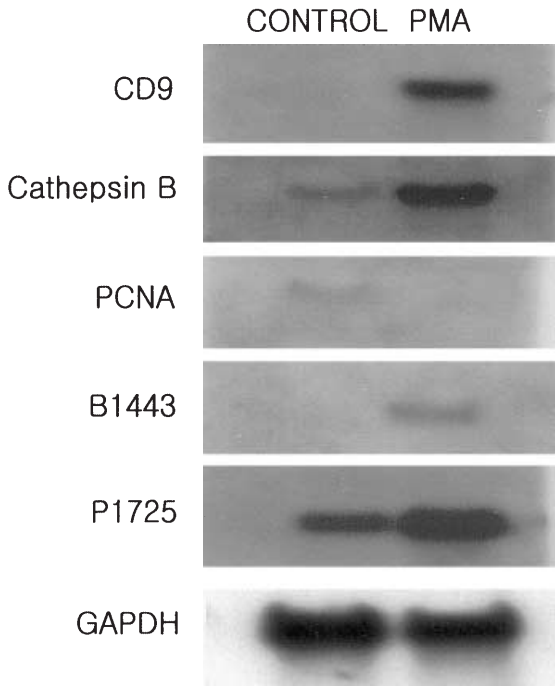

Fig. 3. Northern blot assay of gene expression. Each lane contains 10 μg of total RNA. Control, untreated HL-60 cells; PMA, 48 h after PMA induced differentiation.

Cathepsin B is a lysosomal cysteine protease (Burnett *et al.*, 1983). HL-60 cells were shown to produce cathepsin B in response to treatment with 12-*O*-tetradecanoylphorbol 13-acetate (Burnett *et al.*, 1983), and its production was related to differentiation of the HL-60 promyelocytes into mature macrophage-like cells (Mort and Buttle, 1997). Cathepsin B may be a useful immunohistochemical marker for malignant and non-malignant cells of the monocyte/macrophage lineage (Burnett *et al.*, 1983). It functions in intracellular protein catabolism and in certain situations may also be involved in other physiological processes, such as processing of antigens in the immune response, hormone activation, and bone turnover (Mort and Buttle, 1997). We also confirmed the changes of Cathepsin B expression in the human HL-60 cell line with differentiation by DNA microarray and Northern blot.

In HL-60 cells, increases in the level of the CD9 protein occur in parallel with PMA-induced differentiation. CD9 expression in PMA-treated HL-60 cells is regulated at the transcriptional level and activation of transcription occurs subsequent to the production of proteins induced as an immediate–early response to PMA (Xu *et al.*, 1994). CD9 gene expression was increased during macrophagic differentiation of HL-60 cells (Fig. 4).

Fibrillarin is a histone-like major protein in the nucleolus that was found to be overexpressed in proliferating cells. The level of fibrillarin decreased

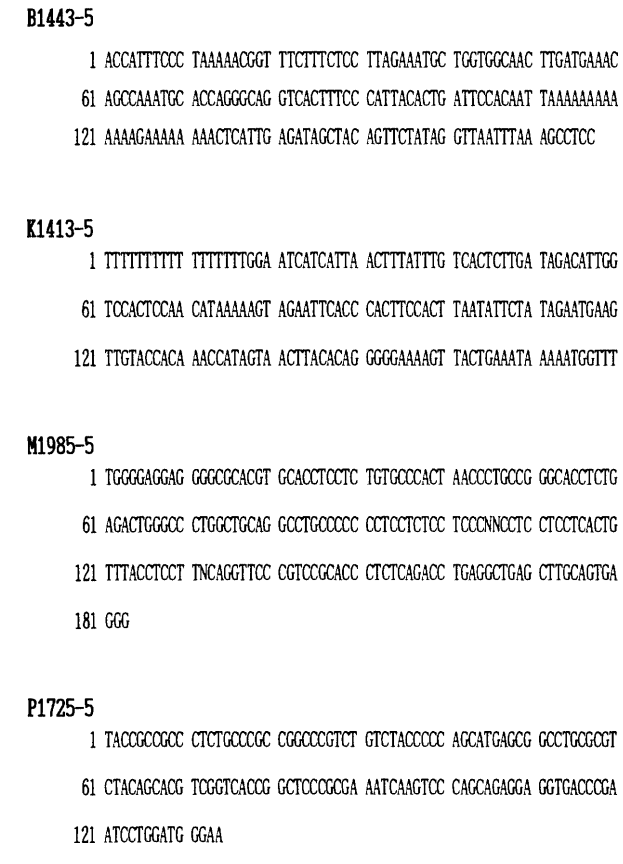

Fig. 4. The nucleotide sequence of four unknown genes which showed increased expression during differentiation of HL-60 cells.

more rapidly and more extensively in differentiating HL-60 cells (Mehes and Pajor, 1995). Fibrillarin RNA expression decreased in PMA-treated HL-60 cells as expected (Table 1).

Galectin-1 is a member of a family of β-galactoside-binding proteins and it has growth regulatory and immunomodulatory activities and its level is increased in activated macrophages (Rabinovich *et al*., 1999). In the present study, galectin-1 is shown to be induced during macrophagic differentiation of HL-60 cells as expected (Table 1).

MGC-24 is a sialomucin originally found in human gastric carcinoma cells and in human hematopoietic progenitor cells (Zannettino *et al*., 1998). CD164 represents a potent signaling molecule with the capacity to suppress hematopoietic cell proliferation (Kurosawa *et al*., 1999). MGC-24 exhibited 5.70-fold induction in this microarray test.

CAPL (also known as S100A4/pEL98/p9Ka/mts1/18A2/42A/calvasculin/FSP1) encoding an S100-related calcium-binding protein is implied to be involved in the invasion and metastasis of tumor cells in murine and human (Lloyd *et al*., 1998; Takenaga *et al*., 1994) and the expression of mts1 was induced during macrophagic or granulocytic differentiation of HL-60 cells in response to PMA or DMSO, respectively (Takenaga *et al*., 1994), and mts1 may be involved in regulating cell motility. The expression of mts1 was induced during PMA-induced differentiation (Table 1).

Treatment of HL-60 cells with phorbol 12- myristate 13-acetate (PMA) results in growth arrest and differentiation towards the macrophage lineage. Among the cell cycle genes identified as targets, the cyclin-dependent kinase inhibitor p21 (WAF1/Cip1) was induced, whereas the proliferating cell nuclear antigen was repressed as expected (Table 1 and Fig. 4). PMA induces rapid activation of the extracellular signal-regulated kinases (ERKs). Activation of the ERK pathway is essential to PMA-induced differentiation of HL-60 cells. PMA also induces the expression of the cyclin-dependent kinase inhibitors p21 (WAF) and p27 (kip1) (Das *et al*., 2000). During PMA-induced differentiation, p21 RNA expression decreased as expected (Table 1).

SGK RNA expression increased during monocytic differentiation (Table 1). SGK is a member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum (Webster *et al*., 1993). The expression of sgk was induced during macrophagic or granulocytic differentiation of HL-60 cells in response to PMA (Lee, 1998). Terminal monocytic differentiation was characterized at the nuclear level by high levels of phosphatidyl inositol 3-kinase and PLC (Neri *et al*., 1999), and SGK was known as a target of the PI3-kinase-stimulated signaling pathway (Park *et al*., 1999). Therefore, SGK may be a component of the PI3-kinase signaling pathway during monocytic differentiation of HL-60.

Three novel genes (B1443, M1985, P1725) which did not have any match in the public data base were also identified. Among these clones, the nucleotide and deduced amino acid sequences of a novel cDNA clone (P1725) are shown in Fig. 5. Nucleotide sequences of the DNA fragment were determined by the dideoxy-nucleotide chain termination method (Kim *et al*., 1998; Sanger *et al*., 1977). Clone P1725 had an insert of 747 bp. The deduced amino acid encodes 93 residues. The cDNA sequence was deposited in GenBank as accession number AF304163.

Microarrays offer a number of advantages over other potential high-capacity approaches to expression analysis. The chip-based approach enables small hybridization volumes and high array densities (Schena *et al*., 1996). Our analysis indicates that the differentiation inducer influences a variety of gene expressions. Further analyses should certainly provide new connections between differentiation-inducer and cellular pathways that cannot be anticipated by our current limited knowledge of the genes controlling proliferation and differentiation.

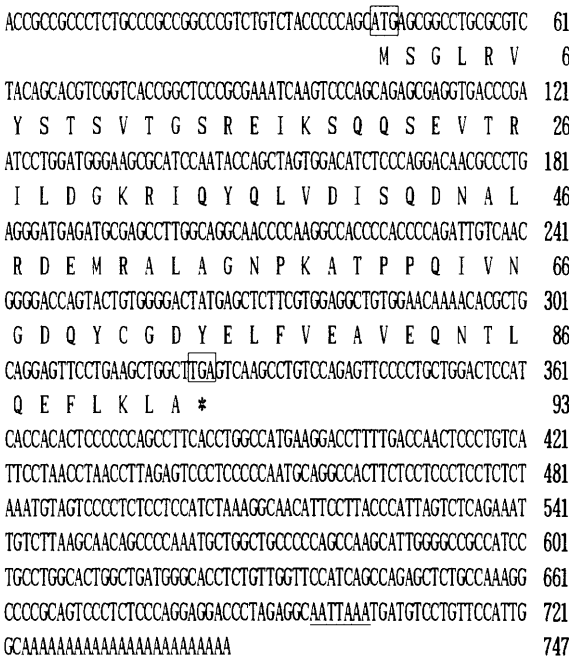

Fig. 5. Complete nucleotide and deduced amino acid sequences of a novel cDNA (P1725). The start and stop codons are marked with the open boxes. The putative polyadenylation signal is underlined. The sequences are deposited in GeneBank (accession no. AF304163).

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