Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 339 (2006) 217–225
BBRC
www.elsevier.com/locate/ybbrc

PKCα expression regulated by Elk-1 and MZF-1 in human HCC cells ☆

Yi-Hsien Hsieh [a], Trang-Tiau Wu [b], Jen-Hsiang Tsai [c], Chih-Yang Huang [a], Yih-Shou Hsieh [a], Jer-Yuh Liu [a,*]

[a] *Institute of Biochemistry and Biotechnology, College of Medicine, Chung Shan Medical University, Taichung 402, Taiwan*
[b] *Department of Surgery, School of Medicine, College of Medicine, Chung Shan Medical University, Taichung 402, Taiwan*
[c] *Department of Nursing, Fooyin University, Kaohsiung 831, Taiwan*

Received 31 October 2005
Available online 10 November 2005

Abstract

Our previous study found that PKCα was highly expressed in the poor-differentiated human HCC cells and associated with cell migration and invasion. In this study, we further investigated the gene regulation of this enzyme. We showed that PKCα expression enhancement in the poor-differentiated human HCC cells was found neither by DNA amplification nor by increasing mRNA stability using differential PCR and mRNA decay assays. After screening seven transcription factors in the putative *cis*-acting regulatory elements of human PKCα promoters, only Elk-1 and MZF-1 antisense oligonucleotide showed a significant reduction in the PKCα mRNA level. They also reduced cell proliferation, cell migratory and invasive capabilities, and DNA binding activities in the PKCα promoter region. Over-expression assay confirmed that the PKCα expression may be modulated by these two factors at the transcriptional level. Therefore, these results may provide a novel mechanism for PKCα expression regulation in human HCC cells.

Keywords: PKCα; Elk-1; MZF-1; Transcription regulation; Antisense oligonucleotide

Protein kinase C (PKC) is an important family of signaling molecules that regulate the proliferation, differentiation, transformation, and apoptosis in cells [1]. The ten PKC isoforms are divided into conventional (cPKCs: α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ), and atypical (aPKCs: ξ and ι/λ) subclasses, depending on their requirement for Ca^{2+}, phosphatidylserine, and diacylglycerol [2]. The individual PKC isoforms differ in their substrate specificity, cellular and subcellular distribution, and tissue specific expression, and it is likely that differential activation of PKC isoforms by second messengers such as diacylglycerol, arachidonic, and phosphoinositides plays a unique role in their regulation and function [3].

Although changes in PKC isoforms are important for the progression in the various cancers [4], the involvement of these enzymes in human HCC remains unclear. Recently our data had found that although eight PKC isoforms were detected in the five human HCC cell lines, only PKCα and PKCι were found to be highly expressed in the poorly differentiated HA22T/VGH and SK-Hep-1 HCC cell lines as compared with the well-differentiated PLC/PRF/5, Hep3B, and HepG2 ones. Antisense oligonucleotides PKCα, but not PKCι, significantly decreased the growth rate in HA22T/VGH and SK-Hep-1 cells by arresting the cell cycle at the G_0/G_1 phase, and also significantly reduced the migratory and invasive capabilities in these cells. These data suggested that PKCα plays an important role in the malignancy of human HCC (submission for publication).

In this study, we further characterized the molecular mechanisms involved in the regulation of PKCα gene expression in the poorly differentiated human HCC cells.

☆ *Abbreviations:* PKC, protein kinase C; Elk-1, Ets-like protein-1; MZF-1, myeloid zinc finger-1; HCC, hepatocellular carcinoma; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ODN, oligonucleotide; FBS, fetal bovine serum; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; β_2-MG, beta-2 microglobulin; RT-PCR, reverse transcription-polymerase chain reaction;

* Corresponding author. Fax: +886 4 23248195.
E-mail address: jyl@csmu.edu.tw (J.-Y. Liu).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.015

The data found that although PKCα expression was not found to be significantly enhanced by DNA amplification or increasing mRNA stability, ElK-1 and MZF-1 transcription factors may be the critical regulators of PKCα expression in the HA22T/VGH and SK-Hep-1 human HCC cell lines.

Materials and methods

Materials. Anti-PKC isoform monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-α-tubulin polyclonal antibodies were brought from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled anti-mouse secondary antibody was obtained from Promega (Madison, WI). All primers and antisense ODNs were provided by MDBio (Taipei, Taiwan) and Mission Biotechnology (Taipei, Taiwan), respectively.

Cell culture. HA22T/VGH (BCRC No. 60168), PLC/PRF/5 (BCRC No. 60223), Hep3B (BCRC No. 60434), and HepG2 (BCRC No. 60025) were purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan) and SK-Hep-1 from the American Type Culture Collection (Rockville, MD). The HA22T/VGH and SK-Hep-1 lines are poorly differentiated, whereas PLC/PRF/5, Hep3B, and HepG2 are well differentiated [5,6]. These cell lines were cultured with DMEM (Gibco-BRL) supplemented with 100 μM non-essential amino acid, 2 mM glutamate, 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Sigma Chemicals, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37 °C.

Extraction of DNA and differential PCR. Genomic DNA was extracted from each cell line by the SDS–proteinase K and the phenol–chloroform extraction method [7]. The differential PCR method was used to reflect the level of target gene amplification [7]. The following oligonucleotide primers were used for PKCα and dopamine D2 receptor (D2R, a reference gene) gene sequence amplification: PKCα, 5′-CCCGGCACCTACCAG ATGAAGTCG-3′ and 5′-CGGCGGAGGCAAGAGGTGGTT-3′ (215 bp fragment 392381–392596, GenBank Accession No. NT_024892); D2R, 5′-CCACTGAATCTGTCCTGGTATG-3′ and 5′-GTGTGGCAT AGTAGTTGTAGTGG-3′. The PKCα DNA fragment sequence was located on extron 1 and intron 1. After confirming the specificity of each primer set, PCRs were performed at the following conditions: 1 cycle of 5 min at 95 °C for DNA (100 ng) template denaturation followed by 35 cycles of 1 min denaturation at 95 °C, 1 min for primers (0.5 μM) annealing at 57 °C, and 2 min for polymerase extension at 72 °C. All PCRs were terminated with a 20 min extension at 72 °C. The PCR products were electrophoresed on 10% polyacrylamide gels and analyzed by direct visualization after SYBR Green I staining. The relative intensities of the PKCα and D2R gene band were compared densitometrically using a Kodak Scientific IU-imaging System. Every PCR was repeated at least twice to confirm the results.

Analysis of mRNA stability. HA22T/VGH and HepG2 cells were treated with 10 μg/ml actinomycin D [8]. Total mRNA was isolated at different times after adding actinomycin D and analyzed for the presence of mRNA for PKCα expression by RT-PCR.

Oligonucleotide synthesis. The ODN primers used in RT-PCR were as follows: PKCα, described previously [9]; β_2-MG as an internal control and described previously [10]; Elk-1: 5′-TTCTCTCAGACAGCTTATCCTT-3′ (sense) and 5′-GCCTAGAATAGAGACAGGACAG-3′ (antisense); MZF-1: 5′-ATAGAATCCCTACCCCTGCATT-3′ (sense) and 5′-CTGA ACACCTTGCCACATACAT-3′ (antisense); Egr-1: 5′-CAAGAGACT TAAAGGACAGGAG-3′ (sense) and 5′-ATGCAAATTATCTTCAC ATCAA-3′ (antisense); GATA-1: 5′-TGACTTTTCCAGTACCTTCTTT (sense) and 5′-TCACCTGGTGTAGCTTGTAGTA (antisense); NF-κB: 5′-TGCATGTGACTAAGAAGAACAT-3′ (sense) and 5′-CAGAGGGT AATAGGTGAACTGT (antisense); AP-2: 5′-TAACATCCCAGATCAA ACTGTA-3′ (sense) and 5′-TTCTCTTAAAGATCTTCCTCCA-3′ (antisense); Oct-1: 5′-CTATGGGGAAACTATATGGAAA-3′ (sense) and 5′-AAGTGCCTGTAACTGAAAGATT-3′ (antisense). The MZF-1, Elk-1, Egr-1, GATA-1, NF-kB, AP-2, and Oct-1 primers were designed using the Biology Workbench 3.2 software (San Diego Super Computer Center, http://workbench.sdsc.edu/).

RNA isolation. Total RNA was isolated from cells by the guanidinium thiocyanate–phenol method [11]. The HCC cell lines were homogenized (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sodium lauryl sarcosinate, and 0.1 M β-mercaptoethanol) in a polypropylene tube, then total RNA was isolated using a standard method. Phenol–chloroform reagent was added to the samples, and centrifuged the tube at 12,000*g* for 30 min at 4 °C, RNA was precipitated from the aqueous phase using isopropanol, and then the resultant pellet was washed twice with 70% ethanol. The RNA content of the resuspended pellet was quantified and checked for purity and condition by spectrophotometry at a wavelength of 260 nm. The extract integrity was assessed by 1.5% agarose gel electrophoresis and RNA was visualized by ethidium bromide staining.

Reverse transcriptase-PCR. RT-PCR assay was performed according to De Petro et al. [12] with slight modifications. An aliquot of total RNA (0.5 μg) was reverse transcribed using 0.5 μM oligo d(T) primers in a reaction solution (50 μl) containing 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, 10 U RNase inhibitor (Promega, Madison, WI), 0.8 mM total dNTPs, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). The sample was incubated at 42 °C for 1 h and at 99 °C for 5 min before chilling on ice for 10 min.

The RT product (2 μl) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris–HCl, and 2 mM $MgCl_2$) to a final volume of 50 μl, containing 0.5 μM dNTPs (final concentration, 0.8 mM) and 0.5 U of Super-Therm *Taq* DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). For each experiment, up to 33 cycles were performed to avoid reaching the PCR plateau values. The PCR products were analyzed by 1.2% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Rockland, ME) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT). The accuracy of the amplification reaction for each set of primers was determined by amplifying several dilutions of the same cDNA with the same cycling profiles and amplifying the same cDNA dilution with different cycling profiles. The specificity of the cDNA was also checked using DNA sequence analysis (data not shown).

Antisense knockout assay. The antisense knockout assay was performed according to Shen et al. [13] and the following antisense and sense (as a control) sequences were used: PKCα (antisense 5′-GTTCTCGC TGGTGAGTTTCA-3′, sense 5′-GGTTTTACCATCGGTTCTGG-3′) [13]; PKCι (antisense 5′-TGTGGGACATGGAGCTGCTG-3′, sense 5′-CAGCAGCACCATGTCCCACA-3′); Elk-1 (antisense 5′-CAGCGTCA CAGATGGGTCCAT-3′, sense 5′-ATGGACCCATCTGTGACGCTG-3′) [14]; MZF-1 (antisense 5′-TACACAAGGGGACCATTCATTC-3′, sense 5′-GAATGAATGGTCCCCTTGTGTA-3′). Egr-1 (antisense 5′-G GGGTAGTTGTCCAT-3′, sense 5′-ATGGACAACTACCCC-3′) [15]; Oct-1 (antisense 5′-GGATTGTTCATTCTTGAGTC-3′, sense 5′-GACT CAAGAATGAACAATCC-3′); NF-κB (antisense 5′-GGATCATCTTC TGCCATTCTG-3′, sense 5′-CAGAATGGCAGAAGATGATCC-3′) [16]; AP-2 (antisense 5′-GTCAATTTCCAAAGCATTT-3′, sense 5′-AAA TGCTTTGGAAATTGAC-3′). The ODN sequence of PKCι is in the region 251–270 of human PKCι mRNA (GenBank Accession No. NM_002740), the MZF-1 ODN sequence is in the region 1089–1110 of human MZF-1 mRNA (GenBank Accession No. M58297), the Oct-1 ODN sequence is in the region 82–101 of the human Oct-1 mRNA (GenBank Accession No. X13403) and the AP-2 ODN sequence is in the region 61–79 of human AP-2 mRNA (GenBank Accession No. X52611). They were formed for targeting the AUG region and had no more than four contiguous intrastrand base pairs or four contiguous G:C pairs.

Cells were plated at 70% density 24 h before antisense ODN treatment. The cells were washed in triplicate with serum-free DMEM and incubated with antisense ODN (0, 0.5, 1.0, 2.0, or 5.0 μM) in serum-free DMEM containing 10 μg/ml lipofectin (Life Technologies, Grand Island, NY) at

37 °C. The medium was changed to 10% FBS DMEM 6 h later before culturing at 37 °C for 24 or 48 h.

Cell proliferation assay. Cell proliferation was determined by the yellow tetrazolium MTT assay [17]. The cells were seeded in 24-well plates at a density of 1×10^4 cells/well and cultured in DMEM containing 10% serum overnight. These cells were treated with and without specific antisense ODNs and incubated for 24 or 48 h. After incubation, the medium was replaced with fresh medium and the cells were incubated with 5 mg/ml MTT for 4 h before dissolving in 1 ml isopropanol for 10 min. The optical density at 570 nm was then measured using a spectrophotometer. Cells at the log phase were used to calculate the doubling time according to the equation doubling time $(h) = [\log\ 2 \times (24 \times \text{No. of days})]/[\log \text{Density}_{\text{final}} - \log \text{Density}_{\text{initial}}]$.

Migration and invasion assays. The cells were treated with indicated sense or antisense ODN (5 μM), detached by trypsinization 48 h later, and then washed in triplicate in serum-free DMEM. For the migration assay [18], the cells were plated at 2×10^5 cells/well in serum-free DMEM in the upper chamber of a 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD), which was plated with the 8-μm pore size polycarbonate membrane filters (Neuro Probe) for 2 h before the cells were added. The cells were then incubated in a humidified 5% CO_2 atmosphere at 37 °C for 6 h in HA22T/VGH cells, for 12 h in SK-Hep-1 cells, and for 24 h in other cell lines. The invasion assay [18] was performed in the same manner as the migration assay, except that the filter was precoated with 10 μg/ml Matrigel (Collaborative Biomedical Products, Bedford, MA) and the cells were incubated at the same conditions for 8 h in HA22T/VGH cells, for 24 h in SK-Hep-1 cells, and for 24 h on other cell lines. After incubation, the cells were fixed with methanol and stained with 0.05% Giemsa. Those on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed in distilled water until no additional stain leached. The cells were then air-dried for 20 min. The number of cells was counted within a field at 200× under a light microscope. For each membrane, a total of 4 fields were selected at random and the numbers were averaged.

Nuclear extracts and electrophoretic mobility shift assays. The cells grown to 80% confluency were used for preparing nuclear extract by a modification of the method of Torgeman et al. [19]. The cells were released from a 60-mm dish with trypsin/EDTA, washed with PBS, and allowed to swell on ice for 10 min in a buffer containing 10 mM Hepes–KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mg/ml leupeptin. Nonidet P40 was then added to a 0.5% final concentration. The suspension was vigorously vortexed for 20 s and briefly centrifuged at 14,000*g* and 4 °C for 15 min. The pellet was resuspended in a buffer containing 20 mM Hepes–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, and 10 mg/ml leupeptin. After vigorously vortexing, the suspension was placed on ice for 15 min before centrifuging 15,000*g* and 4 °C for 15 min. The supernatants (nuclear extracts) were stored in aliquots at −80 °C. Protein concentration of the supernatants was determined by the colorimetric assay (Bio-Rad).

Nuclear extracts and electrophoretic mobility shift assays (EMSA) was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, Illinois). The binding reactions contained 10 μg nuclear extract protein, 10× binding buffer (10 mM Tris (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μg poly(dI-dC), and 2 μM of biotin-labeled DNA. Biotin end-labeled DNA duplex of sequence 5′-CTGAGGATGGGGAAGGG-3′ and nonlabeled oligonucleotide sequences 3′-CCCTTCCCCATCCTCAG-5′ containing a putative binding site for MZF-1 and biotin end-labeled DNA duplex of sequence 5′-CACCGCAGCAGGAAGCCCT-3′ and nonlabeled oligonucleotide 3′-AGGGGCTTCCTGCTGCTGCGGTG-5′ containing a putative binding site for Elk-1 were used. The mixture was incubated at 25 °C for 20 min. After reacting, the DNA–protein complexes were subjected to a 6% native polyacrylamide gel in a 0.5× Tris borate–EDTA buffer at 100 V for 3 h and then transferred onto a positively charged nylon membrane (Hybond-N^+) in 0.5× Tris borate–EDTA buffer at 100 V for 1 h. The membrane was immediately cross-linked for 15 min on a UV transilluminator equipped with 312 nm bulbs and then was detected by chemiluminescence according to the manufacturer's instructions. Supershift EMSA was also performed. The nuclear extracts obtained from HA22T/VGH cells were incubated with biotin-labeled Elk-1 or MZF-1 probe as described above and were also incubated with/without 2 μg of anti-Elk-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-MZF-1 antibody (ABGENT, San Diego, CA), respectively.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation was performed as described [20] with some modifications. The HA22T/VGH cells were treated with indicated sense or antisense ODN (5 μM) and cells were harvested and cross-linked with 1% formaldehyde for 10 min and the reaction was terminated by the addition of 0.25 M glycine. Cells were washed four times in ice-cold PBS, resuspended in RIPA lysis buffer (0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 10 mM $NaPO_4$(pH 7.2), 2 mM EDTA, 0.2 mM $NaVO_3$, and 1% NP-40) in the presence of Complete Protease Inhibitors (Roche Diagnostics, Mannheim, Germany), and sonicated to shear chromatin using a Cole Palmer Ultrasonic processor (Cole Palmer, Vernon Hills, IL). The sonicated DNA fragments were in the range of 100–1000 bp. The samples were pre-cleared with 60 μl protein A–Agarose (Sigma Chemicals, St. Louis, MO) for 30 min at 4 °C. Complexes were immunoprecipitated with 2 μg anti-Elk-1 antibody (Santa Cruz Biotechnology) or anti-MZF-1 antibody (ABGENT). The section of 210 bp PKCα promoter containing the predicted Elk-1 and MZF-1 binding site was detected by PCR with the

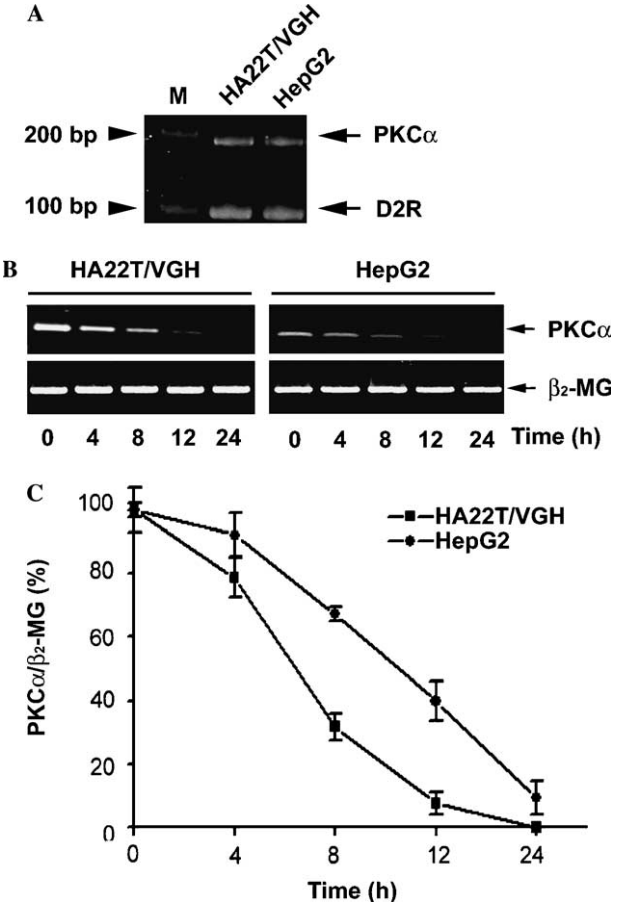

Fig. 1. PKCα gene amplification and mRNA stability detection. (A) Gene amplification of PKCα was determined in HA22T/VGH and HepG2 cells by differential PCR as described in Materials and methods. D2R, dopamine D2 receptor (a reference gene). (B) The decay of PKCα mRNA in the presence of actinomycin D was determined by RT-PCR assay as described in Materials and methods. M, 100 bp ladder as the DNA size marker. (C) Quantities of PKCα mRNA expression in (B). The data represent one of three independent experiments with similar results.

primers 5′-GGTACAGGCAGCTAAAACAC-3′ and 5′-GTCTTCC TTCTCCCACTCC-3′.

Plasmids. The expression vectors described below were driven by the cytomegalovirus (CMV) promoter-basic contained in the pcDNA3 vector (Invitrogen, Carlsbad, CA). The entire open reading frames of human MZF-1 and Elk-1 genes were obtained from the SK-Hep-1 cells by RT-PCR. pcDNA-Elk-1 (GenBank Accession No. NM005229, 234-1520) and pcDNA-MZF-1 (GenBank Accession No. M58297, 1091–2548) were amplified by PCR using the primer pairs 5′-TTATAAGCTTATGGACC CATCTGTGACGCT-3′ encoding nucleotides 234–253 followed by a *Hin*dIII site and 5′-TTATGGATCCTCATGGCTTCTGGGGCCCT-3′ encoding nucleotides 1520–1502 followed by a stop codon (TGA) and a *Bam*HI site, and 5′-TTATAAGCTTTGTCATGAATGAATGGT-3′ encoding nucleotides 1075–1091 followed by a *Hin*dIII site and 5′-TTATC TCGAGCTACTCGGCGCTGTGGA-3′ encoding nucleotides 2532–2548 followed by a stop codon (TGA) and a *Xho*I site, respectively. PCR amplifications were performed with Super-Therm DNA polymerase (Promega) employed 35 cycles with steps at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2.5 min. The PCR products were isolated and cloned into the pGEM-T Easy vector (Promega). pcDNA-Elk-1 was constructed by digesting pGEM-T-Elk-1 with *Hin*dIII and *Bam*HI, and the 1286 bp fragment was isolated and cloned into the *Hin*dIII–*Bam*HI sites of the pcDNA3 vector. pcDNA-MZF-1 was constructed by digesting pGEM-T-MZF-1 with *Hin*dIII and *Xho*I, and the 1457 bp fragment was isolated and cloned into the *Hin*dIII–*Xho*I sites of the pcDNA3 vector. Sequence fidelity of both Elk-1 and MZF-1 was confirmed using DNA sequence analysis (data not shown)

Transfections were performed using lipofectin. Cells seeded at 60-mm dish were cultured for 24 h in DMEM supplemented with 10% FBS, rinsed with serum-free MEM and 1 ml MEM containing 15 μg/ml lipofectin, and 2 or 4 μg pcDNA-Elk-1 or/and pcDNA-MZF-1. The cells were incubated at 37 °C for 6 h before adding 1 ml MEM supplemented with 20% FBS to the medium. After incubation for 18 h, the medium was replaced with fresh 10% FBS–DMEM. Cells were lysed 48 h after transfection with guanidinium thiocyanate buffer for RT-PCR assay and Western blot analysis.

Western blotting. To analyze the expression of individual PKC isoforms, the cultured cells were washed twice with PBS and then lysed with a lysis buffer (50 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40, and 0.3% sodium deoxycholate). The cell lysates were centrifuged at 100,000*g* and 4 °C for 30 min. The supernatant was collected and the protein concentration was determined by the Bradford method. Each sample (40 μg) was then separated by SDS–PAGE using an 10% (w/v) gel and electrophoretically transferred onto a PVDF membrane (Millipore, Belford, MA). The membrane was blocked with 5% (w/v) non-fat dried milk in TBST buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20) and then incubated with the specific anti-PKC isoform (1:1000) or α-tubulin (1:3000) antibody at 4 °C overnight. After washing with TBST, the blots were then incubated with HRP-conjugated anti-mouse antibody (1:3000) at room temperature for 1 h and then washed with TBST before visualized using chemiluminescence (Amersham–Pharmacia Biotech, Piscataway, NJ).

Statistical analysis. The association between the various factors was determined using the Pearson correlation. A value of $P < 0.05$ was considered to be statistically significant.

Results

PKCα expression not enhanced by DNA amplification and increasing its mRNA stability

To determine whether an increase in PKCα expression occurs in HCC cells due to gene amplification, we determined the gene amplification level using the differential PCR method. PKCα gene amplification was not found in HA22T/VGH and HepG2 cells (Fig. 1A). Moreover, we compared the decay of PKCα mRNA in HA22T/VGH

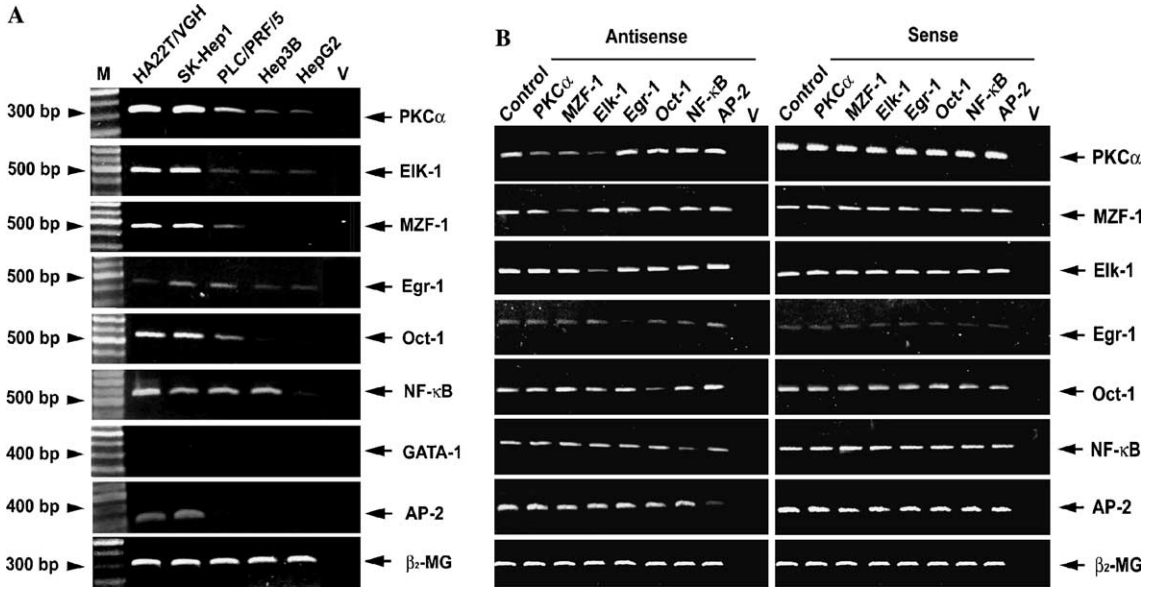

Fig. 2. Expressions of transcription factors in human HCC cell lines. (A) Expressions of PKCα, transcription factors (Elk-1, MZF-1, Egr-1, Oct-1, NF-κB, GATA-1, and AP-2), and β_2-MG in HA22T/VGH, SK-Hep-1, PLC/PRF/5, Hep3B, and HepG2 cells were detected by semiquantitative RT-PCR as described in Materials and methods. V, negative control for PCR without RT; M, 100 bp ladder as the DNA size marker. The data represent one of three independent experiments with similar results. (B) Antisense transcription factors effects on PKCα mRNA in HA22T/VGH cell. After treatment with antisense or scrambled ODN (5 μM) of Elk-1, MZF-1, Egr-1, Oct-1, NF-κB, or AP-2 for 48 h, the mRNA level of PKCα in the HA22T/VGH cells was measured by RT-PCR as described in Materials and methods. V, negative control for PCR without RT. The data represent one of three independent experiments with similar results.

cells with that in HepG2 cells in the presence of actinomycin D to determine whether mRNA stabilization contributed to the elevated PKCα in HA22T/VGH cells. The stability of PKCα mRNA in HA22T/VGH cells was lower than that in HepG2 (Figs. 1B and C). Thus, the high PKCα mRNA level in HCC cells may not be due to gene amplification or the increase in mRNA stability and transcription factor up-regulation may be a plausible mechanism.

The association between PKCα expression and the transcription factors

To determine whether the increase in PKCα mRNA level is a consequence of transcriptional up-regulation, we focused on seven transcription factors in the putative *cis*-acting regulatory elements of human PKCα promoter: Elk-1, MZF-1, Egr-1, Oct-1, NF-κB, GATA-1, and AP-2 [21]. Using RT-PCR, we compared the PKCα mRNA level and the transcriptional factor levels in the five human HCC cell lines. Among these factors, the mRNAs of Elk-1, MZF-1, Egr-1, Oct-1, NF-κB, and AP-2 were detected (Fig. 2A). The Elk-1 ($r = 0.98$), MZF-1 ($r = 0.96$), Oct-1 ($r = 0.95$), NF-κB ($r = 0.69$), and AP-2 ($r = 0.94$) levels were found to be significantly correlated with that of PKCα ($P < 0.01$).

Antisense ODNs transcription factor effects on PKCα expression

To determine whether these transcription factors participated in the up-regulation of PKCα, HA22T/VGH cells were treated with antisense ODNs. Only cell cultures treated with Elk-1 and MZF-1 antisense ODNs for 48 h showed a significant reduction in the PKCα mRNA level (Fig. 2B). Moreover, similar results were also obtained in SK-Hep-1 cells (data not shown).

Antisense ODNs transcription factor effects on the HA22T/VGH HCC cell proliferation

To determine whether inhibition of Elk-1 or MZF-1 mRNA expression affects cell proliferation, cell proliferation assays were performed on the HA22T/VGH HCC cell lines. After 48 h antisense treatment at the 5 μM concentration, the doubling time of cells by antisense Elk-1 was 167.8% of the control cells and antisense MZF-1 156.0%. No cell proliferation inhibition was observed in the other antisense transcription factors (99.5–102.0% of the control). This observation suggests that Elk-1 or MZF-1 has a role in cell proliferation.

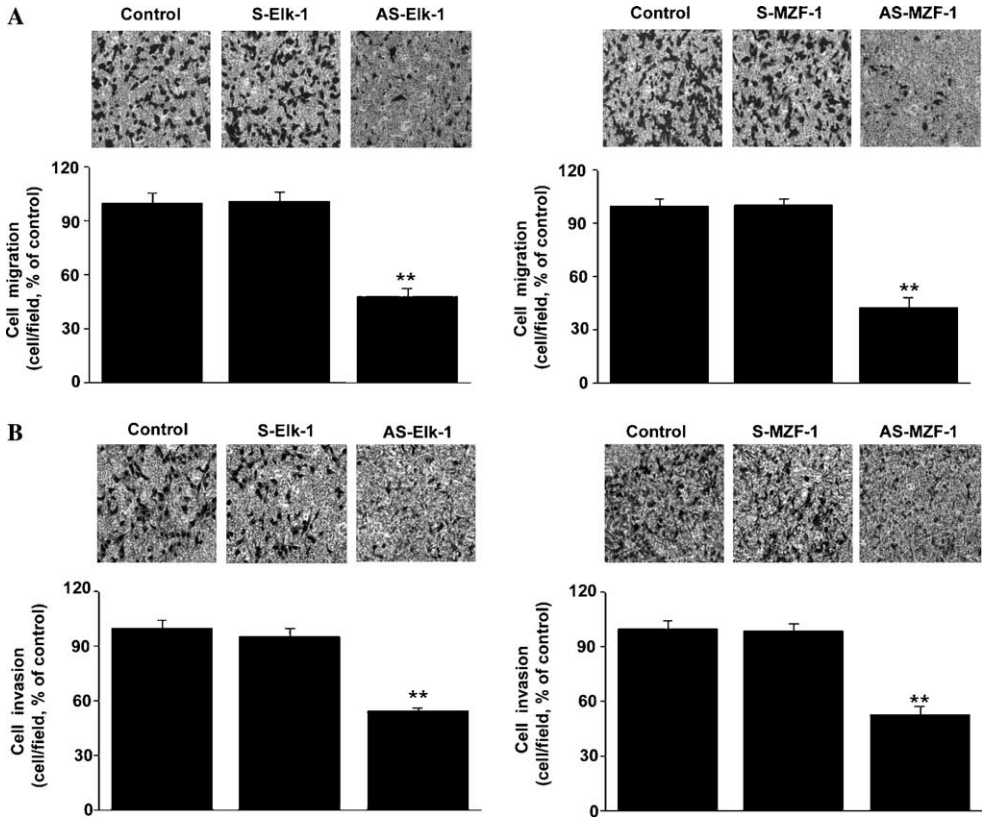

Fig. 3. Antisense Elk-1 and MZF-1 effects on migration and invasion in human HCC cell line HA22T/VGH. The migration (A) and invasion (B) assays were performed on cell cultures treated with antisense Elk-1 (5 μM) (AS-Elk-1) or Elk-1 sense ODN (5 μM) (S-Elk-1), or with antisense MZF-1 (5 μM) (AS-MZF-1) or MZF-1 sense ODN (5 μM) (S-MZF-1) as described in Materials and methods. Untreated cultures were designated as controls (control). $^{**}P<0.01$ as compared to the control.

Inhibition of Elk-1 or MZF-1 impairs migration and invasiveness of HA22T/VGH cells

To determine whether inhibition of Elk-1 or MZF-1 mRNA expression affects cell migration and invasion, migration and invasion assays were performed on the HA22T/VGH HCC cell lines. For invasion assays, the polycarbonate membranes were coated with the reconstitute basement membrane Matrigel. In control cells, the membrane of cells crossing the uncoated filters was about 2-fold to that of cells passing through the Matrigel-coated filters with a longer incubation time indicating that the Matrigel lay constitutes a barrier that had to be actively penetrated by the invasive cells. Antisense Elk-1 inhibited cell migration and invasion by 49% and 42%, respectively, compared with the control or scrambled ODN groups (Figs. 3A and B). Similarly, antisense MZF-1 inhibited cell migration and invasion by 53% and 48%, respectively, compared with the control or scrambled ODN groups (Figs. 3A and B). Thus, it appears that loss of Elk-1 or MZF-1 function inhibits cell migration and invasion.

The binding activities of Elk-1 and MZF-1 to PKCα promoter

To study the binding of Elk-1 and MZF-1 transcription factors to the PKCα promoter region, EMSA was carried out with nuclear protein obtained from HA22T/VGH cells and biotin-labeled double-strand oligonucleotide probes containing the Elk-1 binding site (oligonucleotide) or MZF-1 binding site (oligonucleotide). The binding activities were observed in the control cells (Fig. 4A lanes 2 and 7) and in the Elk-1 or MZF-1 sense-ODN-transfected cells (Fig. 4A lanes 4 and 9), while reduced in the Elk-1 or MZF-1 antisense-ODN-transfected cells (Fig. 4A lanes 5 and 10). The competition reactions were confirmed by adding 200-fold excess nonlabeled double-stranded Elk-1 or MZF-1 consensus oligonucleotide to the reaction mixture (Fig. 4A lanes 3 and 8). To resolve the composition of the Elk-1 and MZF-1 sites, supershift EMSA was performed. Although the supershifted band was not clearly identified, the addition of the antibody of Elk-1 or MZF-1 to the binding reaction dramatically reduced the Elk-1 and MZF-1 (Fig. 4B).

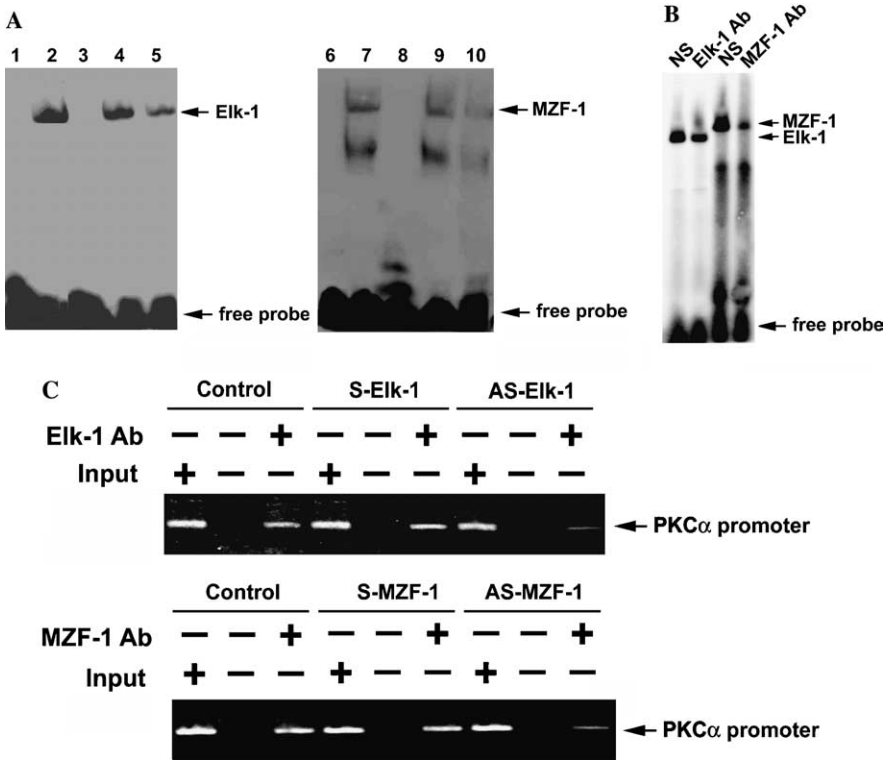

Fig. 4. The binding activities of Elk-1 and MZF-1 to the PKCα promoter. (A) The specific binding activities of Elk-1 or MZF-1 to the PKCα promoter were analyzed by EMSA. Nuclear extracts obtained from HA22T/VGH cells were incubated with biotin-labeled Elk-1 (lanes 1–5) or MZF-1 (lanes 6–7) probe. Lanes 1 and 6, containing labeled probe without nuclear extract; lanes 2 and 7, containing labeled probe with nuclear extract; lanes 3 and 8, containing labeled Elk-1 and MZF-1 probe with nuclear extract and 200-fold molar excess of unlabeled Elk-1 and MZF-1 probe, respectively; lanes 4 and 9, containing labeled Elk-1 and MZF-1 probe with nuclear extract obtained from the Elk-1 and MZF-1 sense ODN-transfected cells, respectively; lanes 5 and 10, containing labeled Elk-1 and MZF-1 probe with nuclear extract obtained from the Elk-1 and MZF-1 antisense-transfected cells, respectively. (B) Supershift EMSA in which nuclear extracts from HA22T/VGH cells were incubated with biotin-labeled Elk-1 or MZF-1 probe in the presence or absence of specific antibodies (Ab) was performed. NS, nonspecific control serum. (C) ChIP assay was performed on HA22T/VGH cells transfected with 5 μM sense or antisense of Elk-1, or with 5 μM sense or antisense of MZF-1. PCR was performed on chromatin fragments enriched by immunoprecipitation with or without the specific antibody (Ab). Input represents the purified input-chromatin for parallel PCR. The data represent one of three independent experiments with similar results.

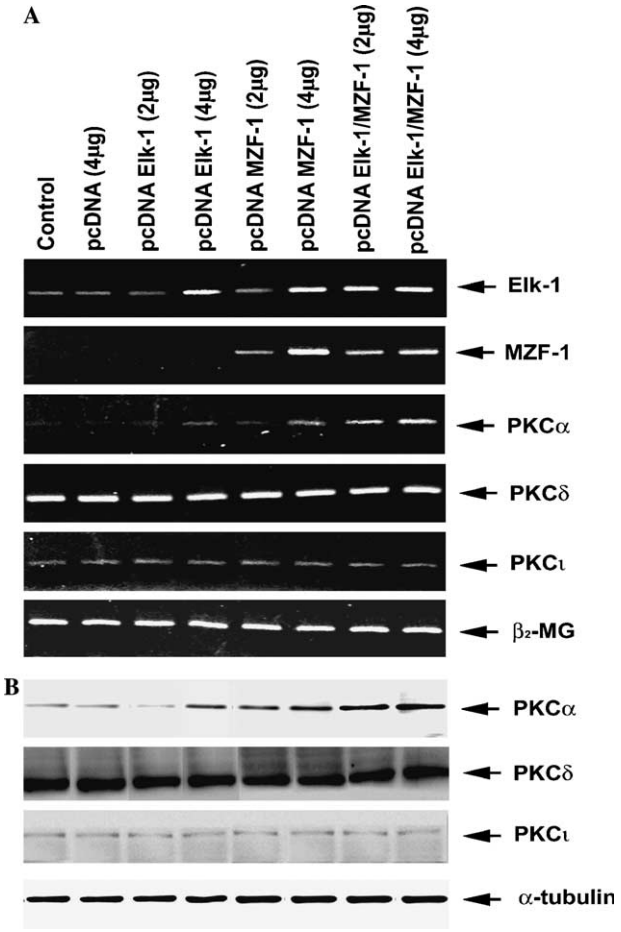

Fig. 5. Over-expressions of Elk-1 and MZF-1 gene effect on the PKCα expression in HepG2 cells. (A) HepG2 cells were transfected with 2 or 4 μg CMV promoter-driven Elk-1 or/and MZF-1 plasmids. The expressions of PKCα, Elk-1, and MZF-1 were measured 48 h after transfection by RT-PCR analysis as described in Materials and methods. Control, untreated cells; pcDNA, transfected only with pcDNA plasmid. (B) The level of PKCα was determined 48 h after transfection by Western blotting as described in Materials and methods. All data represent one of three independent experiments with similar results.

To confirm that endogenous PKCα promoter is bound by endogenous Elk-1 and MZF-1, chromatin immunoprecipitation (ChIP) assays were performed. The result showed that the PKCα promoter fragment was amplified from the immunoprecipitated complex obtained from nuclear extracts incubated with either Elk-1 or MZF-1 antibodies (Fig. 4C). When cells were transfected with antisense Elk-1 or MZF-1, the amplified products of PKCα promoter fragment were reduced, but no change was observed in cells transfected with sense Elk-1 or MZF-1. These findings indicated that Elk-1 and MZF-1 may be directly binding to the promoter region of PKCα in HA22T/VGH cells.

Over-expression of Elk-1 and MZF-1 gene effects on PKCα expression in HepG2 cells

To further examine the role of Elk-1 and MZF-1 genes in PKCα expression, HepG2 cells were transfected with different doses of the CMV-driven human Elk-1 and/or MZF-1 expression vector. The mRNA levels of Elk-1, MZF-1, and PKCα were determined using RT-PCR 48 h after transfection. As shown in Fig. 5A, cells transfected with one gene or both genes showed a dose-dependent Elk-1 and/or MZF-1 expression. HepG2 cells transfected with the Elk-1 and/or MZF-1 vector also showed a dose-dependent PKCα expression. However, the higher level of PKCα expression was observed in the Elk-1 and MZF-1 transfected cells. PKCα expression induced by both Elk-1 and/or MZF-1 over-expression was also confirmed using Western blotting (Fig. 5B). Moreover, after transfected with the CMV-driven human Elk-1 and/or MZF-1 expression vector at the 4 μg concentration each for 48 h, the doubling time decreased from 103.0% for the control cells to 74.9% for Elk-1 and MZF-1 expression vector, but no change was observed in the only Elk-1 or MZF-1 expression vector transfected cells (by 99.0% and 103.1%, respectively).

Discussion

In this study, we found that the great expression of PKCα mRNA in the poor differentiated human HCC cells showed no significant correlation with DNA amplification and mRNA degradation delay. The PKCα gene is located at the chromosomal 17q24 [22] and allelic gain in this region is seldom found in human hepatocellular carcinomas [23]. Our evidences agreed with these findings that DNA amplification of the PKCα gene may rarely occur in human HCC. Moreover, we screened seven transcription factors in the putative *cis*-acting regulatory elements of human PKCα promoter in the human HCC cell lines and found a significant correlation of PKCα mRNA expression with Elk-1 and MZF-1 mRNA expression. Using the antisense knockout assay and ChIP assays, the results suggested that PKCα expression may be regulated by Elk-1 and MZF-1 at the transcriptional level.

In contrast to the transcriptional regulation of PKCα, mutant p53 markedly suppresses PKCα transcription through the Sp1 binding site of PKCα promoter in human leiomyosarcoma cells [24] and in U-87 glioblastoma multiforme cells, *cis* activation of the basal promoter of the human PKCα is predominantly dependent upon the transcription factor AP-2 in a phorbol ester-responsive manner [21]. However, the present data indicated the involvement of a novel mechanism of PKCα gene regulation in human HCC cells.

The interesting finding of this study is the discovery of a functional regulation of human PKCα promoter activity by the transcription factors Elk-1 and MZF-1. Elk-1 is a target of both ERK and JNK MAP kinase cascades [25] and is capable of activating c-*fos* promoter with SRF. Moreover, it has been reported that Elk-1 proteins play a central role in the cell response to extracellular signals and control the expression of genes involved in cell cycle progression, differentiation, and apoptosis [26–28]. Besides, MZF-1 is a transcription factor of the kruppel family of zinc finger proteins, which preferentially expressed in myeloid progenitor cells

[29], and is also involved in the growth, differentiation, and apoptosis of myeloid progenitors [30–32]. Insistently, consensus MZF binding sites were found in the promoter of several hematopoietic cell specific genes, including CD34, c-myc, and lactoferrin [33–35]. However, so far, the function of MZF-1 in transcription regulation was unexplored in other cell types. We demonstrated that both Elk-1 and MZF-1 were highly expressed in human poor differentiated HCC cells and involved in the up-regulation of PKCα, which was essential for cell migration and invasion. Moreover, cells transfected with both Elk-1 and MZF-1 extremely increased the level of PKCα expression, while PKCα was slightly increased in the cells transfected with only either Elk-1 or MZF-1, suggesting the role of these transcription factors in hepatocarcinogenesis possibly in a cooperative manner. Thus, additional studies are therefore required to delineate in greater detail the functional interaction between Elk-1 and MZF-1.

Acknowledgments

We thank Dr. Fen-Pi Chou for her excellent technical assistance and Dr. Jaw-Ji Yang for valuable comments and suggestions. This work was supported by grants from the National Science Council, Republic of China (NSC 92-2745-B-040-001 and NSC 93-2320-B-040-066), and from Chung Shan Medical University, Republic of China (CSMU 92-OM-B-014).

References

[1] A.C. Newton, Protein kinase C: structure, function, and regulation, J. Biol. Chem. 270 (1995) 28495–28498.
[2] S. Ohno, Y. Nishizuka, Protein kinase C isotypes and their specific functions: prologue, J. Biochem. 132 (2002) 509–511.
[3] Y. Shirai, N. Saito, Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting, J. Biochem. 132 (2002) 663–668.
[4] Koivunen J, Aaltonen V, Peltonen J, Protein kinase C (PKC) family in cancer progression. Cancer Lett. (2005) in press.
[5] D.P. Aden, A. Fogel, S. Plotkin, Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line, Nature 282 (1979) 615–616.
[6] C. Chang, Y. Lin, T.W. O-Lee, C.K. Chou, T.S. Lee, T.J. Liu, F.K. P'eng, T.Y. Chen, C.P. Hu, Induction of plasma protein secretion in a newly established human HCC cell line, Mol. Cell. Biol. 3 (1983) 1133–1137.
[7] R.A. Frye, C.C. Benz, E. Liu, Detection of amplified oncogenes by differential polymerase chain reaction, Oncogene 4 (1989) 1153–1157.
[8] L.Y. Romanova, I.A. Alexandrov, R.P. Nordan, M.V. Blagosklonny, J.F. Mushinski, Cross-talk between protein kinase C-alpha (PKC-alpha) and -delta (PKC-delta): PKC-alpha elevates the PKC-delta protein level, altering its mRNA transcription and degradation, Biochemistry 37 (1998) 5558–5565.
[9] K.I. Nagata, Y. Okano, Y. Nozawa, Protein kinase C isozymes in human megakaryoblastic leukemia cell line, MEG-01: possible involvement of the isozymes in the differentiation process of MEG-01 cells, Br. J. Haematol. 93 (1996) 762–771.
[10] O. Takenaka, K. Shichita, M. Tanaka, K. Shuda, M. Yamamoto, Identification and characterization of genes associated with human hepatocellular carcinogenesis, Cancer Res. 59 (1999) 4990–4996.
[11] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[12] G. De Petro, D. Tavian, A. Copeta, N. Portolani, S.M. Giulini, S. Barlati, Expression of urokinase-type plasminogen activator (u-PA), u-PA receptor, and tissue-type PA messenger RNAs in human hepatocellular carcinoma, Cancer Res. 58 (1998) 2234–2239.
[13] L. Shen, N.M. Dean, R.I. Glazer, Induction of p53-dependent, insulin-like growth factor-binding protein-3-mediated apoptosis in glioblastoma multiform cells by a protein kinase C alpha antisense oligonucleotide, Mol. Pharmacol. 55 (1999) 396–402.
[14] W. Yu, Q.Y. Liao, F.M. Hantash, B.G. Sanders, K. Kline, Activation of extracellular signal-regulated kinase and c-Jun-NH(2)-terminal kinase but not p38 mitogen-activated protein kinases is required for RRR-alpha-tocopheryl succinate-induced apoptosis of human breast cancer cells, Cancer Res. 61 (2001) 6569–6576.
[15] M. Pignatelli, R. Luna-Medina, A. Perez-Rendon, A. Santos, A. Perez-Castillo, The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells, Biochem. J. 373 (2003) 739–746.
[16] J.W. Lim, H. Kim, K.H. Kim, Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells, Lab. Invest. 81 (2001) 349–360.
[17] S.B. Sobottka, M.R. Berger, Assessment of antineoplastic agents by MTT assay: partial underestimation of antiproliferative properties, Cancer Chemother. Pharmacol. 30 (1992) 385–393.
[18] J.C. Saurin, M. Fallavier, B. Sordat, J.C. Gevrey, J.A. Chayvialle, J. Abello, Bombesin stimulates invasion and migration of Isreco1 colon carcinoma cells in a Rho-dependent manner, Cancer Res. 62 (2002) 4829–4835.
[19] A. Torgeman, N. Mor-Vaknin, E. Zelin, Z. Ben-Aroya, M. Lochelt, R.M. Flugel, M. Aboud, Sp1-p53 heterocomplex mediates activation of HTLV-I long terminal repeat by 12-*O*-tetradecanoylphorbol-13-acetate that is antagonized by protein kinase C, Virology 281 (2001) 10–20.
[20] C.K. Inman, N. Li, Shore P, Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene beta-casein, Mol. Cell. Biol. 25 (2005) 3182–3193.
[21] J.H. Clark, V. Haridasse, R.I. Glazer, Modulation of the human protein kinase C alpha gene promoter by activator protein-2, Biochemistry 41 (2002) 11847–11856.
[22] Kofler K, Erdel M, Utermann G, Baier G, Molecular genetics and structural genomics of the human protein kinase C gene module. Genome Biol. 3 (2002) research 0014.1–research 0014.10.
[23] G. Wang, Y. Zhao, X. Liu, L. Wang, C. Wu, W. Zhang, W. Liu, P. Zhang, W. Cong, Y. Zhu, L. Zhang, S. Chen, D. Wan, X. Zhao, W. Huang, J. Gu, Allelic loss and gain, but not genomic instability, as the major somatic mutation in primary hepatocellular carcinoma, Genes Chromosomes Cancer 31 (2001) 221–227.
[24] M. Zhan, D. Yu, J. Liu, R.I. Glazer, J. Hannay, R.E. Pollock, Transcriptional repression of protein kinase C alpha via Sp1 by wild type p53 is involved in inhibition of multidrug resistance 1 P-glycoprotein phosphorylation, J. Biol. Chem. 280 (2005) 4825–4833.
[25] K.M. Wiren, A.R. Toombs, X.W. Zhang, Androgen inhibition of MAP kinase pathway and Elk- 1 activation in proliferating osteoblasts, J. Mol. Endocrinol. 32 (2004) 209–226.
[26] A. Khurana, C.S. Dey, Involvement of Elk-1 in L6E9 skeletal muscle differentiation, FEBS Lett. 527 (2002) 119–124.
[27] P. Vanhoutte, J.L. Nissen, B. Brugg, B.D. Gaspera, M.J. Besson, R.A. Hipskind, J. Caboche, Opposing roles of Elk-1 and its brain-specific isoform, short Elk-1, in nerve growth factor-induced PC12 differentiation, J. Biol. Chem. 276 (2001) 5189–5196.
[28] N. Shao, Y. Chai, J.Q. Cui, N. Wang, K. Aysola, E.S. Reddy, V.N. Rao, Induction of apoptosis by Elk-1 and deltaElk-1 proteins, Oncogene 17 (1998) 527–532.
[29] R. Hromas, S.J. Collins, D. Hickstein, W. Raskind, L.L. Deaven, P. O'Hara, F.S. Hagen, K. Kaushansky, A retinoic acid-responsive

human zinc finger gene, MZF-1, preferentially expressed in myeloid cells, J. Biol. Chem. 266 (1991) 14183–14187.
[30] K.A. Robertson, D.P. Hill, M.R. Kelley, R. Tritt, B. Crum, S. Van Epps, E. Srour, S. Rice, R. Hromas, The myeloid zinc finger gene (MZF-1) delays retinoic acid-induced apoptosis and differentiation in myeloid leukemia cells, Leukemia 12 (1998) 690–698.
[31] J.F. Morris, F.J. Rauscher 3rd., B. Davis, M. Klemsz, D. Xu, D. Tenen, R. Hromas, Aberrant expression of the myeloid zinc finger gene, MZF-1, is oncogenic, Cancer Res. 55 (1995) 3610–3614.
[32] L. Bavisotto, K. Kaushansky, N. Lin, R. Hromas, Antisense oligonucleotides from the stage-specific myeloid zinc finger gene MZF-1 inhibit granulopoiesis in vitro, J. Exp. Med. 174 (1991) 1097–1101.
[33] D. Perrotti, P. Melotti, T. Skorski, I. Casella, C. Peschle, B. Calabretta, Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-myb promoter activity, Mol. Cell. Biol. 11 (1995) 6075–6087.
[34] R. Hromas, B. Davis, F.J. Rauscher 3rd., M. Klemsz, D. Tenen, S. Hoffman, D. Xu, J.F. Morris, Hematopoietic transcriptional regulation by the myeloid zinc finger gene, MZF-1, Curr. Top. Microbiol. Immunol. 211 (1996) 159–164.
[35] J.F. Morris, F.J. Rauscher 3rd., B. Davis, M. Klemsz, D. Xu, D. Tenen, R. Hromas, The myeloid zinc finger gene, MZF-1, regulates the CD34 promoter in vitro, Blood 86 (1995) 3640–3647.