Expression of the human cGMP-dependent protein kinase II gene is lost upon introduction of SV40 T antigen or immortalization in human cells

Michihiko Fujii[a], Toshihiko Ogata[b], Eiichi Takahashi[c], Kiyoshi Yamada[a], Kazuhiko Nakabayashi[d], Michio Oishi[a], Dai Ayusawa[d,*]

[a]*Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan*
[b]*National Institute of Health, Toyama, Shinjuku-ku, Tokyo 162, Japoan*
[c]*Otsuka GEN Research Institute, Otsuka Pharmaceutical Co., Ltd., Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan*
[d]*Kihara Institute of Biological Research, Yokohama City University, Maioka-cho, Totsuka-ku, Yokohama 244, Japan*

Received 25 August 1995; revised version received 9 October 1995

Abstract We have cloned a human cGMP-dependent protein kinase type II cDNA to examine its gene expression in terms of cellular senescence and/or immortalization. The genetic locus was mapped to band 4q21 by FISH. Northern blot analysis revealed that expression of the type II gene was markedly decreased or lost in mortal or immortal human fibroblasts producing SV40 T antigen. Also in various immortalized cell lines tested, the gene was not expressed. In normal diploid fibroblasts, the gene was constitutively expressed during cell-cycle and population doubling levels (PDLs).

Key words: cGMP-dependent protein kinase; Gene expression; SV40; Immortalization; FISH; Genetic locus

1. Introduction

Recently, we have shown that the inhibitors of cGMP-dependent protein kinase (cGK) block cellular senescence induced by heat inactivation of thermolabile SV40 T antigen in immortalized human fibroblast cell lines [1]. It is therefore suggested that cGK has some relevance to cellular proliferation since cellular senescence can be taken as one form of growth control.

To date, two types of cGK, typeI which exists as a dimer of identical subunits and and type II which exists as a monomer, have been identified in various organisms. The type I is expressed as two isomers, α and β isoforms, which differ from each other only in the amino-terminal region where dimerization is thought to occur [2–5]. The type I is found predominantly in the cytosol of virtually all cell types, whereas type II is shown to be membrane-bound and localized primarily to epithelial tissues [6,7].The cDNAs encoding both type Iα and Iβ isoforms have been cloned from bovine [8], human [9], *Drosophila* [10], and that encoding the type II has been cloned from *Drosophila* [10], mouse [11], and rat [12]. As regards their functions, only limited roles in specialized biological functions have been reported [1].

By cloning and characterization of the human type II cDNA, we report the mode of expression of the human cGK genes especially in terms of cellular senescence and/or immortalization

*Corresponding author. Fax: (81) (45) 820 1901.

The nucleotide sequence data reported ion this paper will appear in the DDBJ, EMBL, and GenBank nucleotide databases with the following accession number D70899.

2. Materials and Methods

2.1. Cell lines and culture conditions

Mortal lines SVts 6–1 and SVts 9–5, immortal lines SVts 7–1 and SVts 8, each isolated following transfection of human diploid fibroblasts (TIG3) with plasmid pMT-1 encoding thermolabile SV40 T antigen, have been described previously [13,14]. Diploid fibroblast line WI-38, its SV40-transformed immortal derivative VA13 [15], and the other immortal cell lines used were obtained from Japanese Resources Cell Bank (National Institute of Health of Japan). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in plastic Petri dishes except that SVts lines were cultured at their permissive temperature of 35°C as described [16,17].

2.2. RT-PCR amplification

Poly(A)$^+$ RNA was purified from normal diploid fibrolasts (TIG3) using oligo(dT)-latex beads (Takara, Kyoto, Japan) as described [18,19] and reverse-transcribed into first strand cDNA in a mixture containing 50 mM Tris (pH 8.3), 40 mM KCl, 4 mM dithiothreitol, 6 mM $MgCl_2$, 0.1 mM each dNTP, 50 μg/ml random hexamers, 20 U/ml RNase inhibitor (Amersham), 500 ng/20 μl poly(A)$^+$ RNA, and 12 units of AMV reverse transcriptase (RAV-2, Takara) by incubation at 42°C for 1 h. An aliquot of the first strand cDNA was used for the following PCR amplification. The reaction mixtures (10 μl) contained 1 ng of first strand cDNA, 0.5 μM of each oligonucleotide primer, 50 mM KCl, 10 mM Tris (pH 8.4), bovine serum albumin (10 μg/ml), 200 μM each dNTP, and 0.5 units of Taq DNA polymerase (Promega). The reaction profile was denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 3 min at 72°C with 30 rounds of amplification in a model 2000 DNA thermal cycler (Perkin Elmer Cetus).

To amplify a consensus sequence in human cGK II mRNA, two degenerate oligonucleotide primers were used in RT-PCRs.The sense primer was 5'-GARYTIGCIATIYTITAYAAYTG-3' encoding the amino acid sequence ELAILYNC, and the antisense primer was 5'-CCRCARAAIGTCCAIGTYTT-3' encoding KTWTFCG (I: inosine; R: A or G; Y: C or T).

2.3. cDNA cloning and sequencing

PCR products were separated on agarose gels, electroeluted from the gel slices, and were directly cloned into T-vector (Invitrogen). The resulting plasmids were sequenced by the dideoxynucleotide chain termination method with T3 and T7 primers and a modified T7 DNA polymerase (Sequenase,USB kit verII) as described [20]. Several of these clones (1.1 kb products) containing a sequence homologous to that of mouse brain cGKII were used in subsequent analysis.

To isolate a full-length cDNA clone, the above sequence was labeled with [^{32}P]dCTP by random priming and used to screen approximately 1×10^6 recombinant lZapII phage clones containing cDNA prepared from TIG3 cells using a cDNA synthesis kit (BRL choice system) as described [18,19]. The phage clones were converted to pBluescript in vivo and the inserts were subcloned to sequence both strands [20].

SDI 0014-5793(95)01223-0

2.4. Chromosomal localization by direct R-banding FISH

The 2.3 kb cDNA fragment encoding human cGKII was ued to screen approximately 2×10^6 recombinant lDASH phage clones to isolate genomic DNA fragments as described [21]. Purified phage DNAs were used in the direct R-banding fluorescence in situ hybridization (FISH) which is based on FISH combined with replicated prometa-

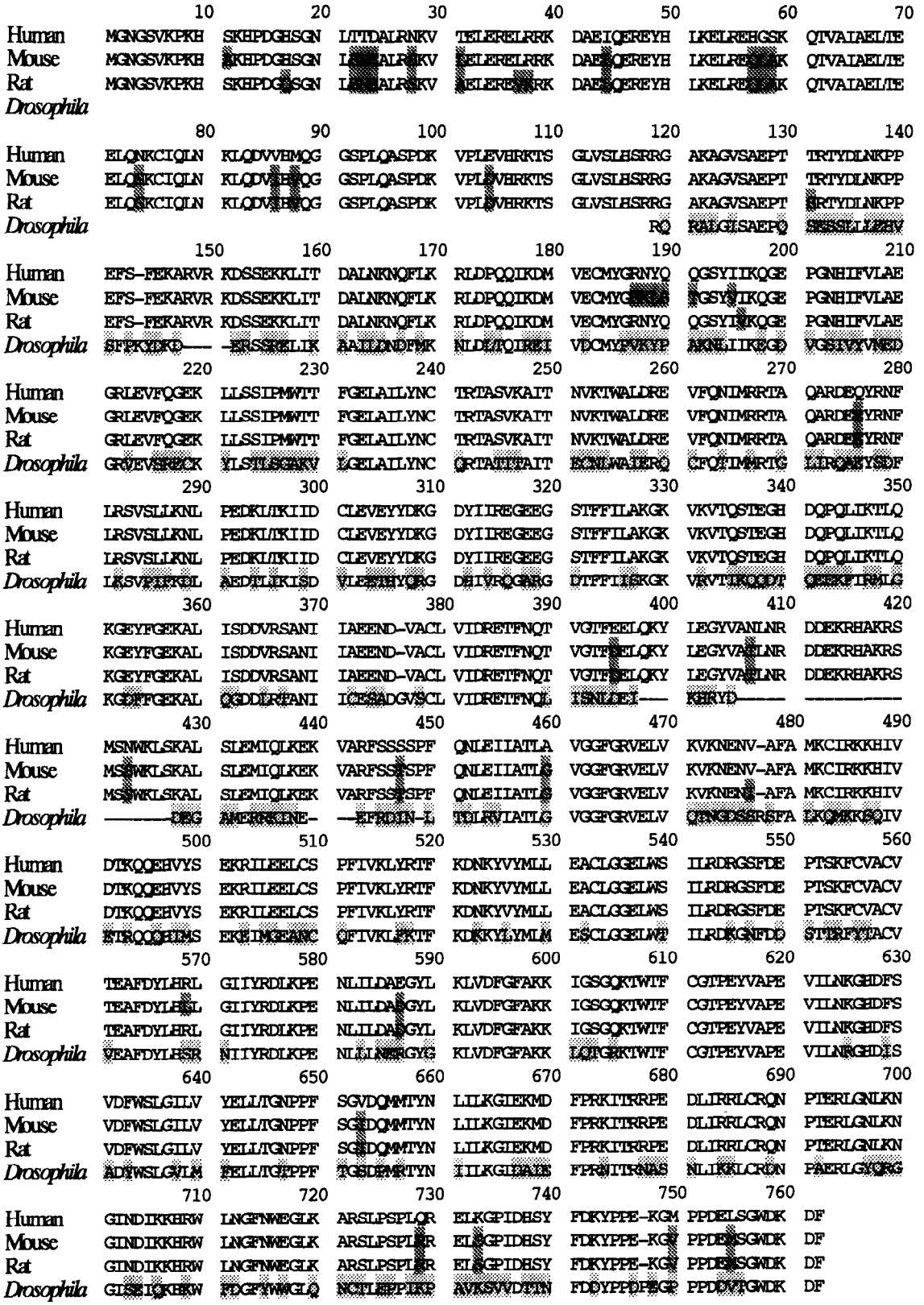

Fig. 1. Alignment of the amino acid sequences of four cGKII species. The human cGKII sequence is compared with mouse brain cGKII, rat intestinal mucosa cGKII, and the deduced cDNA sequence of the *Drosophila* DG2 gene. The N-terminal portion of this cDNA is not shown due to very low sequence homology.

phase R-banded chromosomes as described previously [22]. The R-banded chromosomes were hybridized with biotinylated phage DNA in the presence of human Cot-1 DNA (BRL) and conjugated with fluorescein-labeled avidin. After stained with propidium iodide, microphotographs were taken with filter combinations (Nikon B-2A and B-2E) and Provia 100 film (Fuji, ISO 100).

2.5. Northern blot analysis

RNA samples were resolved by electrophoresis on formaldehyde-agarose gels, transferred onto nylon filters, and hybridized with a ^{32}P-labeled cDNA probe in a mixture consisting of 0.43 M Na-P_i, 7% SDS, 1% bovine serum albumin and 20 mM EDTA (16 h at 38°C). The filters were washed twice in 2 × SSC and 0.1% SDS and twice with 0.1 × SSC and 0.1% SDS at 65°C for 30 min as described [20].

3 Results

3.1. Cloning and characterization of human cGKII cDNA

To clone a member of the human cGK family, we performed RT-PCR using degenerate primers and cDNA made from human fibroblasts. These primers were designed to encode conserved motifs and catalytic domains of *Drosophila* and mouse cGKII sequences, respectively [10,11]. PCR products revealed two major DNA bands of 1.0 kb and 1.1 kb in size on agarose gel electrophoresis [11]. Upon cloning and sequencing of more than 10 clones derived from these two bands, the 1.0 kb DNA band was found to result from human type I cGK sequence [9] and the 1.1 kb band was highly homologous to the type II cGK sequence of mouse brain [11]. No other sequence was detected from the above clones. Using one 1.1 kb clone as a probe, we screened a cDNA library constructed from normal diploid fibroblasts, and isolated two overlapping cDNA clones of 2.3 kb and 1.0 kb in size, which represent the full-length human homolog of the mouse cGKII cDNA.

The nucleotide sequence of the above two clones revealed an open reading frame of 2,286 bp with the first ATG surrounded by an appropriate Kozak consensus sequence [23]. The predicted open reading frame encodes a protein of 762 amino acid residues (87 kDa). The deduced amino acid sequence showed the characteristic domains of the known cGKII proteins [9–11]; i.e. a tandemly duplicated cGMP-binding domain (aa 148–409) followed by a typical protein kinase domain (aa 450–714) (Fig. 1). The human cGKII was compared to other known cGKII. The amino acid sequence was 95.9% and 95.7% homologous to those of rat intestinal mucosa [12] and mouse brain [11], respectively (Fig. 1). The nucleotide sequence was 96.0% and 96.5% homologous to those of mouse and rat species, respectively.

3.2. Genetic locus of the human cGKII gene

To localize the human cGKII gene on human chromosomes, we screened a human genomic library and isolated five independent phage clones which were confirmed to contain a fragment of the cGKII gene by exon mapping followed by partial sequencing.

We examined 100 typical R-banded (pro)metaphase spreads for each 1 phage clone. In one case, 28% exhibited complete twin spots on both homologs, 51% were incomplete single and/or twin spots on either or both homologs, and no spots were detected in the others (21%). All of the five genomic clones gave similar results. The signals were localized to band q21.1-q21.3 on chromosome 4. The gene could therefore be assigned to the band 4q21.1-q21.3 (Fig. 2).

3.3. Gene expression during cell cycle and population doubling levels

We examined expression of the cGKII gene during cell cycle in normal diploid fibroblasts.TIG3 cells were synchronized in G_0 phase of the cell cycle by serum starvation and induced to proliferate by addition of 10% serum [20]. We monitored a 6.0 kb major species of the cGKII mRNA by Northern blot analysis using the 2.3 kb cDNA as a probe.The mRNA level increased slightly (2-fold) during cell cycle progression from G_0 to S phase (Fig. 3A).

Then, we examined the mRNA level in WI-38 cells at their late PDL and a senescent state in culture. In the senesced cells, the mRNA level was increased by 2- to 3-fold (Fig. 3B). In senesced TIG3 cells, however, the mRNA levels was not significantly increased (not shown).

3.4. Gene expression in various cell lines

We examined the cGKII mRNA levels in various cell lines

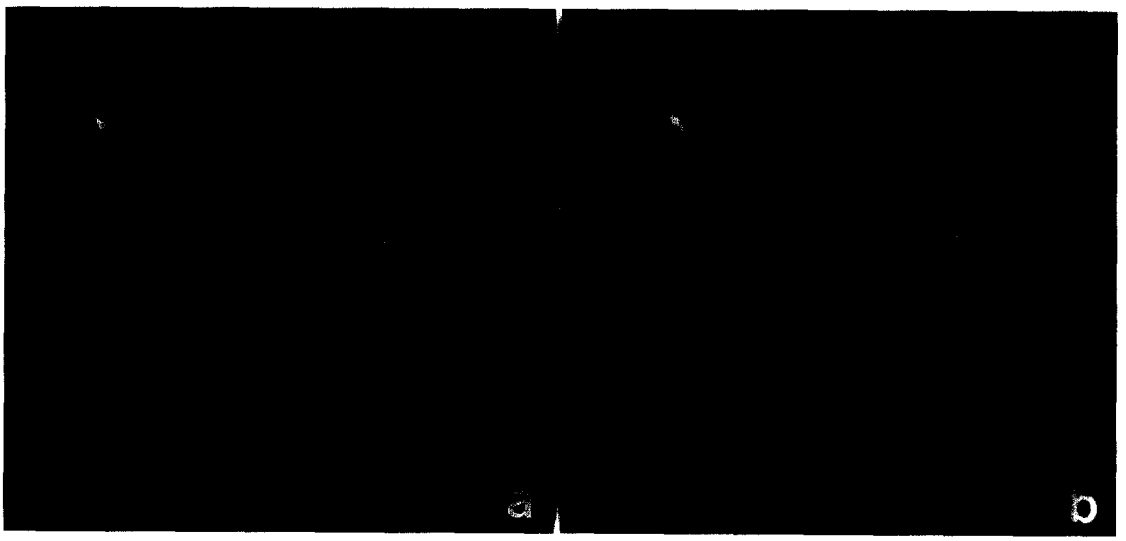

Fig. 2. Whole R-banded metaphase plate (a: B-2A, b: B-2E) after FISH with the human cGKII gene as a probe. Arrows indicate the signals on 4q21.1-q21.3.

by Northern blot analysis. In three normal diploid fibroblast lines TIG1, TIG3 and WI-38, their levels were similar (Fig. 4). In contrast, SVts 6–1 (mortal), SVts 9–5 (mortal), SVts 7–1 (immortal) and SVts 8 (immortal), all derived from TIG3 cells following transfection with a plasmid encoding SV40 large T antigen, showed a marked decrease in their mRNA levels (Fig. 4). The mRNA was also undetectable in WI-38 VA13 (Fig. 4), an immortal SV40-transformed derivative of WI-38, and two SV40-transformed immortal lines, W-V and PSV811, both derived from the fibroblasts of patients with Werner's syndrome (not shown). The loss of expression of the type II gene was confirmed by the more sensitive method of RT-PCR (not shown). In this assay, the cGKII transcript was not detected in the immortal lines SVts 7–1 and SVts 8 and a trace amount was detected in the mortal lines SVts 6–1 and SVts 9–5. These results show that the expression of the cGKII gene is abolished upon introduction of SV40 T antigen regardless of whether cells are mortal or immortal.

We then examined the cGKII mRNA level in other types of immoral lines (not shown). Two immortalized lines KMST-6 and SUSM-1, established in vitro from diploid fibroblasts following repeated γ-irradiation and treatment with 4-nitro-quinoline 1-oxide, respectively, and shown to be free of DNA tumor viruses [16], did not express the cGKII gene. Also, tumor-derived immortal cell lines tested [19,20] such as HT1080 (fibrosarcoma), HeLa (cervical tumor), SAOS-2 (osteosarcoma), HL60 (promyelocytic leukemia) or Y79 (retinoblastoma) did not express the gene.

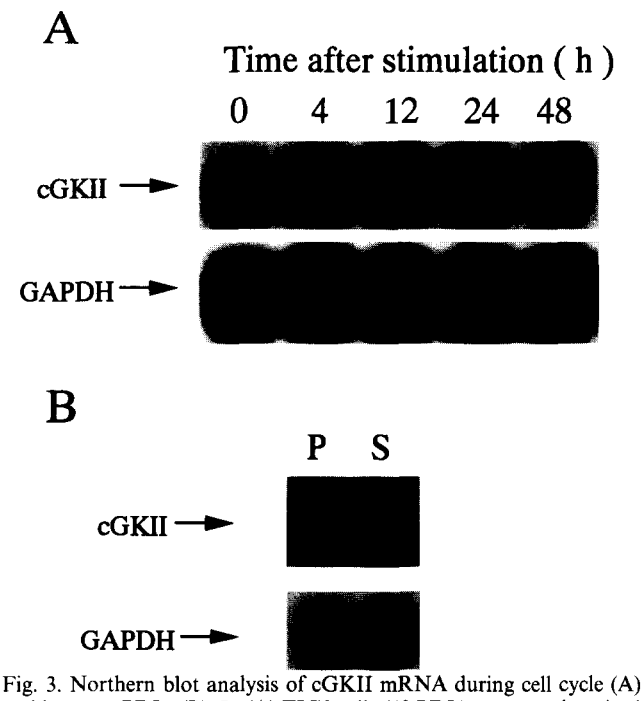

Fig. 3. Northern blot analysis of cGKII mRNA during cell cycle (A) and between PDLs (B). In (A) TIG3 cells (40 PDL) were synchronized in the resting phase by serum starvation and stimulated to proliferate by addition of 10% serum. The cells were then harvested to prepare mRNA samples at intervals as indicated. In (B) WI-38 cells at a late PDL (P: proliferating) and at a senescent state (S: senescence) were harvested to examine the cGKII mRNA.The cells obtained from the Cell Bank at the above PDL enter senescence with additional 21 PDLs. The same blots (2 μg mRNA) were hybridized with the radio-labeled 1.1 kb cGKII cDNA and glyceraldehyde monophosphate dehydorgenase (GAPDH) cDNA as probes.

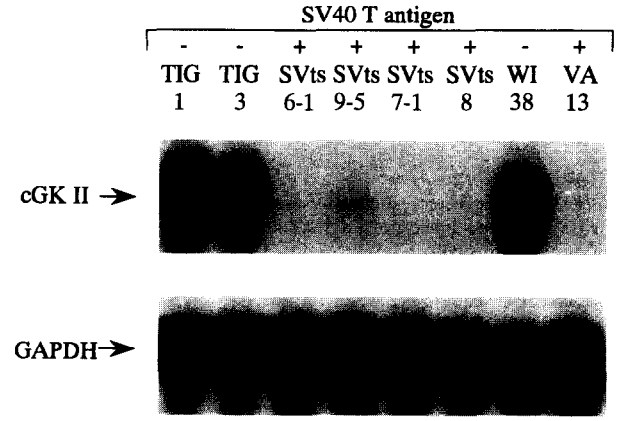

Fig. 4. Northern blot analysis of cGKII mRNA in various cell lines. The same blots (2 μg mRNA) were hybridized as described in the legent to Fig. 3. The samples are indicated above the lanes. Producers and nonproducers of SV40 T antigen are marked with + and −, respectively.

4. Discussion

We have cloned the human cGKII cDNA to examine expression of both type I and type II genes in terms of cellular senescence and/or immortalization. By phylogenetic analysis, the human cGKII cDNA described here is most closely related to the recently cloned counterparts of mouse brain [11] and rat intestinal [12]. The genetic locus of the type I gene (4q21.1-q21.3) is distinct from that of the type Ib gene (10q11.2) [25].

In normal diploid fibroblasts, expression of the cGKII gene seemed to be slightly increased during cell cycle and in senesced cells. However, such changes were not large enough to draw any biological significance. In contrast, the expression of the type II gene varied significantly among cell types. Most remarkably, the expression was abolished in the cell lines expressing SV40 T antigen regardless of whether they were mortal or immortal whereas that of the type I cGK gene did not change significantly. Also in non-tumorigenic immortalized fibroblast lines and several tumor-derived immortalized cell lines tested, the expression was not detected. Therefore, the expression of the cGKII gene seems to be restricted to normal types of cells and to be abolished by introduction of SV40 T antigen or immortalization.

The above results led to suggest that the typeII gene may be regulated by p53 and/or pRB because their functions are inactivated by SV40 T antigen and frequently lost in tumor-derived cell lines. Therefore, we examined the effect of p53 on expression of the cGKII gene using SAOS-2 cells ($p53^-/pRB^-$) transfected with a plasmid encoding temperature-sensitive human p53 [24]. However, the cGKII mRNA was not increased to a detectable level at the permissive temperature where mdm2 and p21 (waf/cip1/sdi1) mRNAs were substantially induced [24].

At present, the biological roles of type I and type II cGK are unknown. Since mortal and immortal types of fibroblasts producing T antigen failed to express the cGKII gene, the loss of its expression is not a specific trait of immortal cells. However, type II cGK might be inhibitory to rapidly proliferating cells since its transcript was so far detected only in normal types of cells.

Acknowledgements: This work was supported by a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, and Culture Japan.

References

[1] Fujii, M., Ide, T., Wadhwa, R., Tahara, H., Kaul, S.C., Mitsui, Y., Oishi, M. and Ayusawa, D. (1995) Oncogene, in press.
[2] Lincoln, T.M., Thompson, M. and Cornwell, T.L. (1988) J. Biol. Chem. 263, 17632–177637.
[3] Wolfe, L., Corbin, J.D. and Francis, S.H. (1989) J. Biol. Chem. 264, 7734–7741.
[4] Lincoln, T.M. and Cornwell, T.L. (1993) FASEB J. 7, 328–338.
[5] Walter, U. (1989) Rev. Physiol. Biochem. Pharmacol. 113, 42–88.
[6] Butt, E., Geiger, J., Jarchau, T., Lohmann, S.M. and Walter, U. (1993) Neurochem. Res. 18, 27–42.
[7] deJonge, H.R. (1981) Adv. Cyclic Nucleotide Res. 14, 315 323.
[8] Wernet, W., Flockerzi, V. and Hofmann, F. (1989) FEBS Lett. 251, 191–196.
[9] Sandberg, M., Natarajan, V., Ronander, I., Kalderon, D., Walter, U., Lohmann, S.M. and Jahnsen, T. (1989) FEBS Lett. 255, 321–329.
[10] Kalderon, D. and Rubin, G.M. (1989) J. Biol. Chem. 264, 10738–10748.
[11] Uhler, M.D. (1993) J. Biol. Chem. 268, 13586–13591.
[12] Jarchau, T., Haeusler, C., Markert, T., Poehler, D., Vandekerckhove, J., DeJonge, H.R., Lohmann, S.M. and Walter, U. (1994) Pro. Natl. Acad. Sci. USA 91, 9426–9430.
[13] Ide, T., Tsuji, Y., Ishibashi, S. and Mitsui, Y. (1993) Exp. Cell Res. 143, 343–349.
[14] Ide, T., Tsuji, Y., Nakashima, T. and S. Ishibashi. 1984.Exp. Cell Res. 150, 321–328.
[15] Girardi, A.J. , Weinstein, D. and Moorhead, P.S. (1966) Ann. Med. Exp. Fenn. 44, 242–254.
[16] Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M. and Oishi, M. (1993) Mol. Cell. Biol. 13, 6036–6043.
[17] Ogata, T., Oshimura, M., Namba, M., Fujii, M., Oishi, M. and Ayusawa, D. (1995) Jpn. J. Cancer Res., 86, 35–40.
[18] Sasaki, Y. F., Ayusawa, D. and Oishi, M. (1994) Nucleic Acids Res. 22, 987–992.
[19] Sasaki, Y.F., Iwasaki, T., Kobayashi, H., Tsuji, S., Ayusawa, D. and Oishi, M. (1994) DNA Res. 1, 91–96.
[20] Kaneda, S., Nalbantoglu, J., Takeishi, K., Shimizu, K., Gotoh, O., Seno, T. and Ayusawa, D. (1990) J. Biol. Chem. 265, 20277–20284.
[21] Yamauchi, M., Yamauchi, N. and Meuth, M. (1990) EMBO J. 2095–2099.
[22] Takahashi, E., D. Ayusawa, Kaneda, S., Itoh, Y., Seno, T. and Hori, T. (1992) Cytogenet. Cell Genet. 59, 268–269.
[23] Kozak, M. (1989) J. Cell Biol. 109, 229–241.
[24] Hirano, Y., Yamato, K. and Tsutida, N. (1995) Oncogene 10, 1879–1885.
[25] Stephens, J. C. (1992) in: Genetic Maps, Cytogenetic Map of Human Genes (O'Brien, S.T., Ed.) Cold Spring Harbor Lab. Press, Plainview , NY, pp. 5.1–5.45.