Isolation and characterization of the human diacylglycerol kinase gene

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The 80 kDa diacylglycerol kinase (DGK) is abundantly expressed in oligodendrocytes and lymphocytes but not to a detectable extent in other cells such as neurons and hepatocytes. As an initial attempt to delineate the mechanism of the transcriptional control of the DGK gene, we have cloned from a human genomic library a 22 kb genomic fragment. The genomic clone consists of the 5'-flanking region and 17 exons coding for approx. 53 % of the total exons of human DGK, including those encoding EF-hand and zinc-finger regions. The translation initiation site is located in the second exon. S1 nuclease mapping and primer extension analysis of the human DGK mRNA identified a major transcription initiation site (position +1) at 264 bp upstream from the initiator ATG. In the 5'-flanking sequence we detected a single GC box at -35 but no canonical TATA and CAAT sequences. However, the sequence starting from the cap site

(AGTTCCTGCCA) is very similar to the initiator element that specifies the transcription initiation site of some housekeeping genes. In addition, the 5'-upstream region contains several putative *cis*-elements. Jurkat and HepG2 cells were transfected with various 5'-deletion mutants of the upstream region fused to the structural gene of chloramphenicol acetyltransferase (CAT). The CAT assay revealed that among constructs containing up to 3.4 kb of the 5'-flanking region, a fragment of 263 bp from the transcription initiation site contains a basic promoter that is active in both types of cells. Moreover, the region between -263 and -850 contains a negative element that is active in HepG2 but not in Jurkat cells. This negative element may, at least in part, be responsible for the cell type-specific expression of the DGK gene.

INTRODUCTION

Diacylglycerol kinase (DGK) converts diacylglycerol into phosphatidic acid. The function of this enzyme in the control of glycerolipid synthesis is unknown, but DGK is thought to play an important role in the signal transduction linked to phospholipid turnover [1], in view of the biological activities of both diacylglycerol [2] and phosphatidic acid [3–5]. Biochemical and immunological studies have shown that animal cells contain several DGK isoenzymes [6]. The molecular sizes of the DGKs so far purified to near homogeneity from animal sources are known to be variable: 80 kDa in porcine brain [7] and thymus [8], and in human white blood cells [9], 150 kDa in porcine thymus [8], 110 and 150 kDa in rat brain [10], and 58, 75 and 152 kDa in human platelets [11]. Furthermore, previous work has shown the presence of a membrane-bound arachidonoyl-diacylglycerol-specific DGK [12,13] and of an isoenzyme exhibiting the same chromatographic behaviour as that of rat brain synapsin I, a basic protein [14]. Although the structural relationships between these different forms of DGK have not been adequately identified, the findings suggest that animal DGK consists of a family of isoenzymes with considerably different properties.

The DGK isoenzyme of approximate molecular mass 80 kDa (80 kDa DGK) was the first to be sequenced by the molecular cloning of cDNA libraries from porcine thymus [15] and later from human white blood cells [9]. The sequences of the two DGK homologues are more than 90 % identical and contain interesting functional domains. The function of zinc-finger structures in the DGK sequence remains ambiguous, but EF-hand motifs [16] have recently been shown to be the high-affinity calcium binding sites of DGK [17,18]. Immunological studies

[19,20] and Northern blotting analysis [9,15,21] previously showed that the 80 kDa DGK has a limited occurrence in animal cells and tissues, being highly expressed in lymphocytes and brain but essentially absent from cells such as hepatocytes and platelets. Moreover, in a recent study in which the rat homologue of 80 kDa DGK was cloned [21], we found by *in situ* hybridization and immunohistochemistry that, within the brain, the expression of this isoenzyme is confined to oligodendrocytes and is notably absent from neurons. In the developmental stage, both DGK mRNA and protein levels gradually increased after birth and reached a peak 3 weeks later, followed thereafter by a gradual decline [21]. Such a developmental pattern of expression suggested that this enzyme may be involved in the maturation process of myelin sheath.

The cell type-specific and developmental stage-dependent patterns of the expression of 80 kDa DGK clearly indicate the importance of transcriptional control of the DGK gene. As an initial step in delineating the regulatory mechanisms of DGK gene transcription, we report here the isolation of the human DGK genomic clone, identification of the transcription initiation site, determination of a part of the exon/intron structures, and detection of the promoter activity in the 5'-flanking region using transient expression of the transfected DGK–chloramphenicol acetyltransferase (CAT) fusion gene. This is the first study at the genomic level of the enzyme involved in the animal glycerolipid biosynthesis.

MATERIALS AND METHODS

Materials

Restriction enzymes, myeloblastosis virus reverse transcriptase, S1 nuclease, T4 polynucleotide kinase and Klenow fragment of

Abbreviations used: DGK, diacylglycerol kinase; CAT, chloramphenicol acetyltransferase.

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The nucleotide sequence data reported have been submitted to the DDBJ Nucleotide Sequence Database under accession no. D16440.

Figure 1 Restriction map of the 5'-end of the human DGK gene and strategy of the construction of DGK-CAT fusion genes

The 5' sequence of human DGK gene is shown by solid lines, with the CAT gene as an open box. The first exon is shown as a filled box.

DNA polymerase I were purchased from Takara Shuzo Co. (Kyoto, Japan). Acetyl-CoA used for the CAT assay was obtained from Wako Pure Chemicals Co. (Osaka, Japan). [γ - 32 P]ATP and [α - 32 P]dCTP were bought from Amersham, and [14 C]chloramphenicol was from du Pont-New England Nuclear. The CAT expression plasmids pUC-CAT [22] and pSV2-CAT [23] were gifts from Dr. K. Fujinaga (Cancer Research Institute, Sapporo Medical College). The β -galactosidase-containing plasmid pCH110 was obtained from Pharmacia-LKB Biotechnology, Inc., and the lipofectin reagent kit was from Gibco BRL. The DNA sequencing kit was obtained from United States Biochemicals. Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer. All other chemicals were of the highest quality commercially available.

Isolation and characterization of genomic clones

General manipulation of DNA was carried out using the standard protocol [24]. A phage EMBL3 library generated by partial digestion of human leucocyte DNA with *Sau*3AI was kindly donated by Dr. Y. Sakaki (Institute of Medical Science, University of Tokyo). Approx. 5×10^6 plaques were screened by plaque hybridization. *Escherichia coli* C600 strain was infected with recombinant phage and replica-plated on nitrocellulose filters. The filters were denatured, neutralized, baked at 80 °C for 2 h, and hybridized with random-primed λ DGK1 clone (2454 bp), which contains nearly the full length of cDNA coding for the porcine DGK (from +38 to +2491) [15]. The hybridization was done for overnight at 60 °C in $3 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate), $10 \times$ Denhardt's solution, 0.1 % SDS and 500 μ g/ml salmon sperm DNA. After hybridization, the filters were washed three times in $0.1 \times$ SSC containing 0.1 % SDS for 30 min at 45 °C, and autoradiographed at -70 °C with intensifying screens. Two positive clones were obtained and restriction mapped. The three *Eco*RI fragments from clone hgDGK4 were subcloned into pBluescript II SK+ (Stratagene) for further analysis. A series of fragments was generated by the *Exo*III/mung bean nuclease method [25]. The exon-containing fragments were first identified by Southern blotting analysis using the porcine cDNA clones λ DGK1 and pDGK23 (358 bp, from -281 to +77) [15] as probes. Exon/intron boundaries were then determined by comparing the sequences of cDNAs of the porcine [15] and human [9] 80 kDa DGKs with that of human genomic DNA, and also by applying the GT/AG rule for the exon/intron border [26]. DNA sequencing of both strands was done by the dideoxy chain-

termination method [27] using the M13 universal and reverse sequence primers.

S1 nuclease protection mapping and primer extension

Total RNA from human thymus (obtained with informed consent from patients undergoing surgical operation for Fallot's tetrad at the College Hospital) was isolated by the guanidine isothiocyanate/CsCl method [28]. The 359 bp *Eco*RV/*Xba*I fragment (-263 to +96 relative to the transcription initiation site) was 32 P-labelled using T4 polynucleotide kinase and used as a probe for S1 nuclease mapping. The labelled probe (100000 c.p.m.) and 50 μ g of the RNA were coprecipitated with ethanol and dissolved in 20 μ l of the hybridization buffer containing 80 % (v/v) formamide, 400 mM NaCl, 40 mM Pipes (pH 6.4) and 1 mM EDTA. After denaturation at 85 °C for 10 min, the mixture was incubated at 51 °C overnight. The mixture was then added to 200 μ l of the buffer containing 250 mM NaCl, 50 mM sodium acetate (pH 4.6), 4.5 mM ZnSO₄, 100 μ g of denatured salmon sperm DNA and 60 or 200 units of S1 nuclease. The digestion was done at 37 °C for 30 min, and the products were analysed on an 8 M urea/6 % polyacrylamide sequencing gel.

For primer extension analysis, a 20-base oligodeoxynucleotide (5'-ATTAAGGCTCTTGGGGTTCG-3') complementary to nucleotides +88 to +69 was synthesized as a primer. After labelling the 5'-end with [γ - 32 P]ATP and T4 polynucleotide kinase, the primer (100000 c.p.m.) and 50 μ g of the human thymus RNA were coprecipitated with ethanol. The precipitate was dissolved in 20 μ l of the buffer containing 10 mM Tris/HCl (pH 8.3), 300 mM KCl and 1 mM EDTA. The mixture was denatured at 85 °C for 10 min, and then allowed to cool slowly to 50 °C. After incubating the mixture for 4 h at 50 °C, the volume was adjusted to 80 μ l by the addition of suitable stock solutions to achieve final concentrations of 20 mM Tris/HCl (pH 8.3), 10 mM MgCl₂, 90 mM KCl, 10 mM dithiothreitol, 0.25 mM EDTA, 250 μ M of each dNTP, 100 μ g/ml actinomycin D and 240 units/ml avian myeloblastosis virus reverse transcriptase. The reaction was carried out for 2 h at 42 °C. The extended products were analysed as described in the S1 mapping experiment.

Plasmid construction and CAT assay

The 5'-flanking region of the DGK gene was isolated from the pBluescript clone, which contained at the *Eco*RI site an insert of about 5.5 kb of the 5'-flanking sequence including the first exon

Figure 2 Structural organization and restriction map of portions of the human DGK gene

The restriction map of the two genomic clones of the human DGK gene is shown at the top (E, *EcoRI*; B, *BamHI*; S, *SalI*). The locations of the 17 exons determined by Southern blotting and sequence determination are indicated as the filled boxes, of which non-coding regions are striped. The structure of the human DGK cDNA is schematically shown according to [9].

(Figure 1). The clone was cleaved by *ApaI* (blunt-ended)/*AvrII*, *HindIII*/*AvrII* and *EcoRV*/*AvrII*, yielding respectively fragments of 1.6 kb (~ -1600 to $+19$), 0.9 kb (-850 to $+19$) and 0.3 kb (-263 to $+19$). The fragments were inserted into the *SalI* (blunt-ended)/*XbaI* or *HindIII*/*XbaI* site of pUC-CAT to generate pDcat 1.6, pDcat 0.9 and pDcat 0.3 respectively. In addition, the 2.5 kb fragment (~ -3400 to -850) was excised by digesting the Bluescript clone with *HindIII* and inserted into the *HindIII* site of pDcat 0.9, thus generating pDcat 3.4. The reverse-oriented pDcat 0.3 (pDcat -0.3) was prepared by insertion of a blunt-ended 0.3 kb (*EcoRV*/*AvrII*) fragment into the blunt-ended *SalI*/*XbaI* site of pUC-CAT. pUC-CAT was digested with *SalI*/*XbaI*, self-ligated and used as the low-background negative control vector. pSV2-CAT, having the CAT gene under the control of the simian virus 40 early region promoter, was used as the positive control vector.

Prior to DNA transfection, the human T cell line Jurkat and the human hepatoma cell line HepG2 were cultured in RPMI 1640 medium and minimal essential medium respectively, each being supplemented with 10% fetal bovine serum. Jurkat cells (6×10^6 cells) were transfected with the fusion gene and pCH110 plasmid (5 μ g of each) using the lipofectin reagent kit according to the manufacturer's instructions. HepG2 cells (2×10^6 cells/100 mm plate) were transfected with the fusion gene and pCH110 (10 μ g of each) by the standard calcium phosphate precipitation protocol described by Sambrook et al. [24]. After 48 h, cells were harvested and cellular extracts were assayed for CAT activity as described [23]. The incubation was continued for 6 h at 37 °C, and the quantification of the CAT activities was achieved by liquid scintillation counting of the acetylated [14 C]chloramphenicol separated by t.l.c. [24]. The differences in transfection efficiencies were corrected for by assaying the β -galactosidase activity.

RESULTS

Isolation and structure of the human DGK gene

Comparison of the amino acid sequences of the porcine [15] and human [9] homologues of 80 kDa DGK showed that they are more than 90% identical. We anticipated that the porcine cDNA clones could serve as probes for screening the human genomic library. We thus screened a human EMBL3 genomic library with

a 32 P-labelled λ DGK1 clone coding for nearly the entire length of the porcine DGK cDNA ($+38$ to $+2491$) [15]. We isolated from 5×10^6 plaques two clones designated hgDGK1 and hgDGK4, spanning 13 and 17 kb respectively. The two clones were restriction-mapped and were found to overlap. The combined restriction maps are shown in Figure 2. The hgDGK4 clone was selected for further analysis since this clone was hybridized with the porcine pDGK23 clone (from -281 to $+77$), indicating the presence of the 5'-flanking region. The three *EcoRI* fragments from hgDGK4 were subcloned into pBluescript II SK+, extensively mapped by restriction enzyme analysis, and the sequences were determined. The exon/intron boundaries were determined by comparing the sequence of the porcine DGK cDNA and the genomic DNA, and also by applying the characteristic GT/AG rule for the nucleotides flanking the exon border [26]. The DNA sequences coding for the human and porcine DGKs are highly similar even in the non-coding region. The 5'-flanking sequences in the first (183 nucleotides) and the second (81 nucleotides) exons were about 80% identical to the 5'-non-coding sequence determined for the porcine DGK cDNA [15]. Furthermore, the non-coding nucleotide sequence in the second exon and the first five nucleotides in the first exon determined in this work were identical to the human cDNA sequence [9] obtained from the EMBL data bank, thus confirming the exon/intron boundary determined for the two exons. However, the remaining upstream 17-base non-coding nucleotide sequence of the human cDNA was completely different from that of the first exon, for unknown reasons.

The hgDGK4 clone was found to contain 17 exons ranging in size between 74 and 183 bp, as summarized in Table 1. We could not detect additional exons in the hgDGK1 clone. The translation initiation site was located at the second exon. The coding region corresponds to about 65% of the porcine cDNA, and the deduced amino acid sequence (474 residues) was identical to that described for the human cDNA [9]. Based on the size of the human DGK mRNA estimated by Northern blot analysis (3.2 kb) [9; and results not shown], the length of the exons determined in the present work (1687 bp) accounts for about 53% of total exons. The 80 kDa DGK is known to contain functional domains such as EF-hand motifs and zinc fingers in its N-terminal portion [9,15,21]. Each of the two zinc fingers is encoded by two exons (exons 9/10 and exons 11/12), whereas the

Table 1 Exon/intron organization of the human DGK gene

Nucleotides in exons are given as capital letters and those in introns as lower-case letters. Codon phases are indicated by boxing.

Exon	3' acceptor	Exon size (bp)	5' donor	Preceding or interrupted amino acid
1		183	CAG	gtaaag
2	gtcacctttccccag	GCC 145	AAT	gtgagt Tyr-22
3	ctctctacaccctag	ACT 74	GAT	gtgagt Asp-46
4	tcactctcattatag	GCC 136	AAG	gtatgg Asp-92
5	cttttttgtaaccag	ATG 75	AAT	gtgagt Phe-117
6	taaaacacccacag	TCA 50	TCA	gtgagt Ser-133
7	cccttttgctccag	GAA 75	CCG	gtaagg Pro-158
8	ccactctgtgtccag	ATT 120	ATG	gtgagt Met-198
9	tctcatgcctcctag	ACC 115	AOC	gtagta Leu-237
10	ttccttctccctcag	TCT 89	GGT	gtgagt Gly-266
11	gcctttctccccaag	GTC 120	GAG	gtcagt Glu-306
12	cacctcgtctttcag	ATC 99	CTG	gtgaga Leu-339
13	tctagccctccccag	GCC 84	CGG	gtacag Arg-367
14	cttcccccaatgcag	ATT 74	GAG	gtgagg Arg-392
15	ttttccacacacag	GST 75	AGG	gtgagc Gly-417
16	tccccttggctcag	GCT 86	TTC	gtagtg Asp-446
17	caactgcctccgcag	ACA 87	GAG	gtaagt Gly-474

Figure 3 Determination of the transcription initiation site of the human DGK gene

(a) S1 nuclease mapping. Total human thymus RNA (Th) or yeast tRNA (Y) was hybridized to the 5'-end labelled S1 probe (−263 to +96) and digested with 60 or 200 units of S1 nuclease, as indicated. The size of the protected bands was determined using a sequencing ladder generated using M13 primer and pBluescript II SK+. (b) Primer-extension analysis. Total human thymus RNA (Th) or yeast tRNA (Y) was hybridized to an end-labelled 20-mer antisense oligonucleotide beginning 88 nucleotides downstream from the transcription initiation site shown in Figure 4. The lengths of the extension products were determined by electrophoresis on a sequencing gel with a sequence ladder, which was generated using the same oligonucleotide primer and the DGK gene (*EcoRV/XbaI* fragment) as a template.

first EF-hand motif is encoded by three exons (exons 5–7) and the second by a single exon (exon 8) (Figure 2).

Determination of the transcription initiation site

To identify the transcription initiation site, we first performed S1 nuclease mapping analysis using a probe (−263 to +96 bp) excised from the hgDGK4 clone by *EcoRV/XbaI* treatment. A major 96-nucleotide fragment was protected by the human thymus mRNA but not by yeast RNA (Figure 3a). Two minor bands of 86 and 84 nucleotides were also protected when treated with the lower concentration of S1 nuclease. To confirm the results obtained from S1 nuclease mapping analysis, we next carried out primer extension analysis. In this case, a 20-mer synthetic oligonucleotide encoding the anti-strand sequence from positions +88 to +69 was used as a primer. One major elongation product extending to position +1 was detected (Figure 3b). Of the two minor fragments detected in the S1 nuclease mapping analysis, the 86-nucleotide band may be significant since a minor extension product at +11 was also detected. Other minor extension products were considered insignificant in view of the results of S1 mapping analysis. The results obtained by S1 nuclease mapping and primer extension analyses indicated the same major transcription initiation site. We, therefore, concluded that the transcription initiation site is the A residue located 264 bp upstream of ATG, which is located in the second exon. However, we also found evidence for the presence of a minor initiation site at position +11.

The nucleotide sequence of the 5'-flanking region of the cap site, the first exon and a part of the second exon is presented in Figure 4. In the 5'-flanking region, we did not find the canonical TATA and CAAT boxes at their usual locations relative to the transcription initiation site. There are two TATA-like sequences at −200 (TATATAT) and −334 (TATATTT), but they are considered to be non-functional because of their unusual location. We noted, however, the presence of a GC box at −35 (GGGGCGGGG). Furthermore, the sequence starting from the cap site, AGTTCCTGCCA, is very similar, with one mismatch

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-850  aagctttgctaacagtgaagaaaactgtaagacaggatggggagtggtcaagggcctgttttttttttttttttttttttagacagagtt
-750  ttgctcgtgtcaccagggtggagtgagtgccatgctctcggtcactgcaacctctgcctcccaggttcaagcgattctcctgcctcagcctcgag
-650  aactgggactacaggcatgtgcaccacgcccggctaattttttgtatttttagtagagacaggttttaccgtgttagcaggatgtctcaatctctg
-550  tctcgtgatccgctgcctcagcctgcccagtgctgggattacagtgtagccacgcgccggccgtttggcctgtctttttatgattgggggtga
-450  aggggtgagacttgaggggcacaggagcagagagacagaccctctgcaacctatgaccccgaaatctccagtttgatatacaacaataactcccaag
-350  cctattactcagcagctatatctctggcaaatgtggaggagtgctgttccaacaagcctgtcttgaaacaatcaagaactgcttagatatccctgttccc
-250  ctagaggccctcatgtattcattccctttccaacccccacaaaagtatgatataatgtgtgtgtatgtatgcatacacaataccacacatgcat
-150  tctcccctcccccttcttccaccccagggtgtaagaaggacgattccaggaacctaaaggctttgtactctgccccattttcaatgaagggtaggagg
-50   agttagataggggtggggcggggcagtgagcaggatgctgggtctccagttcctgccaagtgagtccttaggcctccatctcttcccttgctgtac
51    ACCTTACCACCATCCATGCGACCCCAAGAGCCTTAATGACTCTAGAAGAGACTCCAGGCAGGGGAAGCTGAAAGGACCTTCACTCCCTACTTTGGC
151   AGGGCCTTCTGTGCCACCTGCCAAGACCAGCAGgtaaatgg... ~5.1 kb ...ctttcccagGCCTACCCTCTGAAGAGGTCCAAGCAACGGAAG
218   ACTACTACGAAGCTGCCTTTCTGGCCATCCTTGAGAAAAATAGACAGATG...

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Figure 4 Nucleotide sequence of the 5'-flanking region of the human DGK gene including exons 1 and 2

The nucleotide sequence of the 5'-flanking region and a part of the first intron is presented as lower-case letters, and nucleotides of exons are given as capital letters. Bases are numbered (indicated on the left of the sequence) with respect to the major transcription start site (designated +1), which is marked with an arrow. The initiator ATG codon in the second exon is shown in bold. Putative regulatory elements are boxed and include the binding sites for HIP1 (housekeeping initiator protein 1) at +1, SP-1 at -35, c-ets oncoprotein at -18 and -571, AP-2 at -127, a metal responsive element at -494, and TATA-like sequences at -200 and -334. A stretch of 28 T residues starting at -789 is underlined.

(underlined), to the initiator element (ATTCN₁₋₃₀GCCA) that defines the transcription initiation site of several housekeeping genes, such as the dihydrofolate reductase gene [29,30]. There are several consensus sequences, each with a one-base mismatch indicated by the underlining: a metal responsive element (CGCGCCCGGCC) [31] at -494, an AP-2 binding site (CCCCAGGT) [32] at -127, and two binding sites (at -18 and -571) for c-Ets-1 oncoprotein (CAGGATGC/T) [33]. We also noted a long stretch of 28 T residues starting at -789.

Promoter activity of the 5'-flanking region

In order to see whether the 5'-flanking region contained a functionally active promoter, we constructed various CAT expression plasmids containing fragments ranging from 0.3 to 3.4 kb of the 5'-upstream sequences, with a constant 3'-end at position +19. The CAT constructs were transfected to Jurkat T cells, which have been known to express a high level of DGK mRNA [9]; and results not shown) as well as of enzyme protein [20]. As shown in Figure 5(a), pDcat 0.3, which contains 263 bp of the 5'-flanking region, showed promoter activity. On the other hand, the same flanking region ligated in the reverse orientation relative to the CAT gene (pDcat -0.3) gave only background activity. The ligation of constructs containing nucleotides further upstream of the 5'-flanking region, up to 3.4 kb, did not considerably affect the CAT activity. The results show that a basic promoter activity can be expressed by the first 263 bp of the 5'-flanking sequence. In the next experiment, the fusion genes were transfected to HepG2 cells (Figure 5b), which do not contain detectable levels of DGK mRNA or enzyme protein. pDcat 0.3 showed a promoter activity in this cell line also, suggesting that the promoter is not cell type-specific. However, in contrast to Jurkat cells, the constructs containing longer inserts produced a markedly reduced level of *in vitro* CAT activity in HepG2 cells, as summarized in Figure 5(c). The results suggest that the region between -263 and -850 contains a negative element that is active in HepG2 cells but not in Jurkat cells.

DISCUSSION

Among the various DGK isoenzymes so far characterized, the mRNA and enzyme protein of 80 kDa DGK are known to be highly abundant in lymphocytes and oligodendrocytes, but are not detectable in other types of cells such as neurons, hepatocytes and platelets. Furthermore, the expression of this enzyme is subject to developmental control in rat brain [21]. The characterization of the genomic structure is essential for understanding such cell-specific and development-dependent patterns of expression of this enzyme. It may be also of interest to determine the exon/intron structures of the gene encoding EF-hand motifs and zinc fingers, both of which are highly conserved in a variety of other proteins. Such knowledge may provide a clue as to the evolutionary relationships among the widely conserved domains.

In the present study, we have cloned and characterized the 5'-flanking region and a part of exon/intron structures which correspond to approx. 53% of the total length of the human DGK cDNA. The 5'-flanking region lacks the canonical TATA and CAAT boxes within 100 bp upstream of the transcription initiation site. The absence of TATA and CAAT boxes at their usual positions relative to the cap site indicates that the DGK gene is a housekeeping gene, in spite of its markedly cell-specific expression. In many housekeeping genes without TATA and CAAT boxes, transcription starts at multiple sites [34]. However, the DGK gene appears to have a single major transcription start site at 264 bp upstream from ATG (approx. 5.3 kb upstream on the gene), although there was also a possible minor transcription start site at position +11. Among the housekeeping genes, the genes encoding dihydrofolate reductase and several other proteins have the initiator element (ATTCN₁₋₃₀GCCA) at the site of transcription initiation [29,30]. The combination of at least one GC box and this initiator element is sufficient for specifying the transcription initiation site of such housekeeping genes [30]. It is likely that the cap site sequence of the human DGK gene from position +1 (AGTTCCTGCCA) in com-

Figure 5 CAT activity in the extracts of Jurkat and HepG2 cells transfected with the fusion gene

Jurkat (a) and HepG2 (b) cells were transfected with the fusion gene constructs by lipofection and calcium phosphate precipitation methods respectively. After 48 h cells were harvested and, after normalization for β -galactosidase activity, assayed for CAT activity. The result is a representative CAT assay from three repeated transfections. Lane 1, pDcat 3.4; lane 2, pDcat 1.6; lane 3, pDcat 0.9; lane 4, pDcat 0.3; lane 5, pDcat -0.3; lane 6, pSV2-CAT (positive control). (c) Following quantification for acetylation of chloramphenicol, the activity with pDcat 0.3 was arbitrarily designated 100%, and relative activity is presented for both cell lines. The results are means $\pm$ S.D. from three independent transfections for each cell line.

bination with a single GC box at -35 bp accounts for transcription initiation at a single major site.

The promoter activity of the 5'-flanking region was established by measuring transient expression of a fusion CAT reporter gene transfected to Jurkat and HepG2 cells. In Jurkat cells, which are known to contain a high level of the DGK mRNA [9], the 5'-flanking sequence corresponding to -263 to +19 had a distinct promoter activity in the *in vitro* CAT assay. This flanking region showed promoter activity even when transfected to HepG2 cells, which contained no detectable DGK mRNA in Northern blot analysis (results not shown). However, we detected the presence of a negative *cis*-element(s) that can be involved in the suppression of gene transcription in hepatocytes. This negative element does not appear to be active in Jurkat cells (Figure 5c), thus suggesting

a mechanism which allows the efficient transcription of this gene in lymphocytes. It is not clear at the present stage whether cells expressing abundantly the DGK gene contain transcriptional factors which interact with the *cis*-elements of this gene. There remains a possibility that, in addition to the promoter activity and the negative element(s) we detected, positive *cis*-elements are required to achieve the cell type-specific expression of the DGK gene. However, the 5'-flanking region of the DGK gene does not appear to possess such positive *cis*-elements, since upstream 5' sequence up to -3.4 kb did not significantly affect *in vitro* CAT activity in Jurkat cells. It is therefore possible that the DNA elements conferring the cell type-specific positive regulatory activity may be located in the further 3'-downstream sequence of this gene, which includes the first intron, spanning approx. 5.1 kb. Such a location of regulatory elements is unusual but has been described for several genes, including those encoding platelet-derived growth factor [35], collagen II [36], 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [37] and others. In any case, further studies are required to fully account for the cell-specific expression of the DGK gene.

We determined the sequence of exon/intron boundaries which correspond to the N-terminal 474 residues of human DGK. The positions of introns in the EF-hand motifs are completely different from those found in genes coding for EF-hands of calmodulin [38] and calcium-dependent neutral proteinase [39]. This may suggest that the evolutionary origin of EF-hands of DGK may be different from that of other EF-hand-type calcium binding proteins. The basic structure of the zinc fingers in DGK is conserved in several proteins, such as protein kinase C [40], *n*-chimaerin [41] and the *vav* proto-oncogene product [42]. The two fingers of DGK are each encoded by two exons (exons 9/10 and exons 11/12). The positions of introns are between Asn-236 and Leu-237, and between Glu-306 and Ile-307, in the first and second fingers respectively. The evolutionary significance of these intron/exon positions awaits genomic analysis of other related zinc-finger proteins.

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REFERENCES

- 1 Kanoh, H., Yamada, K. and Sakane, F. (1990) Trends Biochem. Sci. **15**, 47-50
- 2 Nishizuka, Y. (1984) Nature (London) **308**, 693-698
- 3 Yu, C.-L., Tsai, M.-H. and Stacey, D. W. (1988) Cell **52**, 63-71
- 4 Tsai, M.-H., Yu, C.-L. and Stacey, D. W. (1989) Science **243**, 522-525
- 5 Moritz, A., DeGraan, P. N. E., Gispens, W. H. and Wirtz, K. W. A. (1992) J. Biol. Chem. **267**, 7207-7210
- 6 Kanoh, H., Sakane, F. and Yamada, K. (1992) Methods Enzymol. **209**, 162-172
- 7 Kanoh, H., Kondoh, H. and Ono, T. (1983) J. Biol. Chem. **258**, 1767-1774
- 8 Sakane, F., Yamada, K. and Kanoh, H. (1989) FEBS Lett. **255**, 409-413
- 9 Schaap, D., de Wit, J., van der Wal, J., Vandekerckhove, J., van Damme, J., Gussow, D., Ploegh, H. L., van Blitterswijk, W. J. and van der Bend, R. L. (1990) FEBS Lett. **275**, 151-158
- 10 Kato, M. and Takenawa, T. (1990) J. Biol. Chem. **265**, 794-800
- 11 Yada, Y., Ozeki, T., Kanoh, H. and Nozawa, Y. (1990) J. Biol. Chem. **265**, 19237-19243
- 12 MacDonald, M. L., Mack, K. F., Williams, B. W., King, W. C. and Glomset, J. A. (1988) J. Biol. Chem. **263**, 1584-1592
- 13 Lemaire, R. N., King, W. C., MacDonald, M. L. and Glomset, J. A. (1990) Biochem. J. **266**, 291-299
- 14 Kahn, D. W. and Besterman, J. M. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6137-6141
- 15 Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C. and Tanabe, T. (1990) Nature (London) **344**, 345-348
- 16 Kretsinger, R. H. (1979) Adv. Cyclic Nucleotide Res. **11**, 1-26

- 17 Sakane, F., Yamada, K., Imai, S. and Kanoh, H. (1991) *J. Biol. Chem.* **266**, 7096–7100
- 18 Sakane, F., Imai, S., Yamada, K. and Kanoh, H. (1991) *Biochem. Biophys. Res. Commun.* **181**, 1015–1021
- 19 Yamada, K. and Kanoh, H. (1988) *Biochem. J.* **255**, 601–608
- 20 Yamada, K., Sakane, F. and Kanoh, H. (1989) *FEBS Lett.* **244**, 402–406
- 21 Goto, K., Watanabe, M., Kondo, H., Yuasa, H., Sakane, F. and Kanoh, H. (1992) *Mol. Brain Res.* **16**, 75–87
- 22 Gilman, M. Z., Wilson, R. N. and Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 4305–4316
- 23 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- 24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 25 Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* **33**, 103–119
- 26 Mount, S. M. (1982) *Nucleic Acids Res.* **10**, 459–472
- 27 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 28 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- 29 Chen, M.-J., Shimada, T., Moulton, A. D., Cline, A., Humphries, R. K., Maizel, J. and Nienhuis, A. W. (1984) *J. Biol. Chem.* **259**, 3933–3943
- 30 Means, A. L. and Farnham, P. J. (1990) *Mol. Cell. Biol.* **10**, 653–661
- 31 Karin, M., Haslinger, A., Heguy, A., Dietlin, T. and Cooke, T. (1987) *Mol. Cell. Biol.* **7**, 606–613
- 32 Imagawa, M., Chiu, R. and Karin, M. (1987) *Cell* **51**, 251–260
- 33 Waslylyk, C., Gutman, A., Nicholson, R. and Waslylyk, B. (1991) *EMBO J.* **10**, 1127–1134
- 34 Martini, G., Toniolo, D., Vulliamy, T., Luzzato, L., Dono, R., Viglittio, G., Pronessa, G., D'Urso, M. and Persico, M. G. (1986) *EMBO J.* **5**, 1849–1855
- 35 Franklin, G. C., Donovan, M., Adam, G. I. R., Holmgren, L., Pfeifer-Ohlsson, S. and Ohlsson, R. (1991) *EMBO J.* **10**, 1365–1373
- 36 Horton, W., Miyashita, T., Kohno, K., Hassel, J. R. and Yamada, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8864–8868
- 37 Lange, A. J., Espinet, C., Hall, R., El-Maghrabi, M. R., Vargas, A. M., Miksicek, R. J., Granner, D. K. and Pilkis, S. J. (1992) *J. Biol. Chem.* **267**, 15673–15680
- 38 Nojima, H. and Sokabe, H. (1987) *J. Mol. Biol.* **193**, 439–445
- 39 Emori, Y., Ohno, S., Tobita, M. and Suzuki, K. (1986) *FEBS Lett.* **194**, 249–252
- 40 Kikkawa, Y., Kishimoto, A. and Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58**, 31–44
- 41 Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P. and Lim, L. (1990) *Biochem. J.* **272**, 763–773
- 42 Coppola, J., Bryant, S., Koda, T., Conway, D. and Barbacid, M. (1991) *Cell Growth Differ.* **2**, 95–105