Genomic structure and transcriptional regulation of the human NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase gene

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Abstract

The NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH) is a catabolic enzyme that controls the biological activities of prostaglandins by converting them into inactive keto-metabolites. Here we report the genomic organisation of the complete human PGDH gene and characterise its transcriptional regulation. The PGDH gene spans about 31 kb on chromosome 4 and contains 7 exons. Within 2.4 kb of the 5'-flanking sequence we identified two regions with clustered putative transcription factor binding sites. The distal promoter element PGDH-DE (positions -2152/-1944 relative to the start codon) contains binding sites for Ets and activating protein-1 (AP-1) flanked by two cAMP-responsive element-binding protein binding sites (CREB1, CREB2), whereas the proximal element PGDH-PE (-235/-153) includes an Ets and an AP-1 binding sequence. By electrophoretic mobility shift assay, no high affinity binding of Ets or AP-1 factors was observed with PGDH-PE, whereas we confirmed interaction of members of the Ets, AP-1 and CREB families of transcription factors with PGDH-DE. Transcriptional control of the PGDH promoter was assessed by transiently transfecting JEG-3 choriocarcinoma cells. A luciferase reporter gene construct containing the PGDH-PE was not induced by c-jun/c-fos in the absence or presence of co-expressed Ets-1. A construct carrying the PGDH-DE in front of the minimal homologous promoter was activated by co-transfection of expression vectors for AP-1 proteins. Mutation of the AP-1 or CREB2 site reduced the response to c-jun/c-fos, whereas mutation of the Ets site of the distal element reduced basal promoter activity. CREB activated the PGDH-DE construct through the CREB1 site. These results defined the distal element as an integrator of transcriptional regulation by AP-1, Ets and CREB proteins.

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Introduction

NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (type-I 15-PGDH; EC 1.1.1.141) plays an important role in the control of the biological activities of prostaglandins (PG). PGDH catalyses the first step of PG catabolism, the oxidation of the 15-hydroxyl group, which results in a reversible inactivation of PGs into their 15 keto-metabolites (Ensor & Tai 1995, Okita & Okita 1996). An absolute requirement for PG metabolism by PGDH in perinatal development has been revealed by the recent generation of PGDH-deficient mice.

Pgdh^{-/-} pups die shortly after birth because of defective remodelling of the ductus arteriosus (Coggins *et al.* 2002). In the human utero-placental unit, PGs are produced by the amnion, chorion and decidua, and the activity of PG synthetases increases at the time of labour (Okazaki *et al.* 1981). It has been suggested that the high level of PGDH expression in the chorion protects the myometrium from biologically active PGs during pregnancy to maintain a quiescent uterus (Mitchell *et al.* 1993, Patel & Challis 2001). PG inactivation is greatly reduced only at the time of parturition, because of diminished PGDH activity (Van Meir *et al.* 1997b,

Pomini *et al.* 2000). Patients in preterm labour have markedly reduced levels of PGDH activity and PGDH mRNA in chorionic trophoblasts (Sangha *et al.* 1994, Van Meir *et al.* 1997a), and PGDH activity is reduced in the myometrium of women in labour, both at term and preterm (Giannoulis *et al.* 2002). Down-regulation of PGDH expression thus appears to play a main role in the initiation of labour at term or preterm. As preterm birth is the predominant cause of neonatal mortality, the prevention of preterm labour is a major goal in obstetrics (López Bernal & Watson 1992). A better understanding of the regulation of PGDH expression may contribute to improved clinical management of this condition.

PGDH cDNA has been cloned from human placenta, mouse lung, guinea pig uterus and rat intestine (Ensor *et al.* 1990, Matsuo *et al.* 1996, Bracken *et al.* 1997, Zhang *et al.* 1997). At present the genomic organisation of the PGDH gene is only known in mice (Matsuo *et al.* 1997). The human gene is located on chromosome 4 (Pichaud *et al.* 1997). Based on the high homology between human and mouse PGDH cDNAs, we devised a long extension PCR strategy to amplify the introns predicted from the structure of the mouse gene and report here the genomic structure of human PGDH.

We have recently isolated 3.5 kb of the 5'-flanking region of the human PGDH gene and reported the presence of several potential binding sites for transcription factors including activating protein-1 (AP-1), Ets and cAMP-responsive element-binding protein (CREB) within 2.4 kb of the 5'-flanking region (Greenland *et al.* 2000). AP-1 belongs to the family of basic region/leucine zipper transcription factors and consists of homo- or heterodimers of Jun, Fos or activating transcription factor (ATF) proteins (Karin *et al.* 1997). Jun-Fos dimers bind to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-responsive elements, while Jun-ATF dimers preferentially bind to the cAMP-responsive element (CRE) (Karin *et al.* 1997). Unlike AP-1, members of the Ets family of transcription factors bind to their target sequence as monomers, but share with AP-1 an involvement in a wide range of biological processes including cellular proliferation, differentiation, transformation and apoptosis (Li *et al.* 2000). In fact, Ets proteins exert many of their activities by protein-protein interactions with other nuclear factors including AP-1 (Li *et al.* 2000, Chinenov & Kerppola 2001).

We have previously demonstrated an involvement of Ets and AP-1 in the transcriptional regulation of PGDH promoter constructs extending to 2.4 kb upstream of the transcriptional start site. Here we analyse more closely two clusters of response elements in the distal and proximal regions of the promoter.

Materials and methods

Isolation and cloning of intronic PGDH DNA

To amplify the intronic regions of the human PGDH gene, ten sense and antisense oligonucleotide primers to the human PGDH cDNA, flanking the splicing sites predicted from the mouse PGDH cDNA, were synthesised (Table 1). Intervening sequences were amplified by PCR using 250 ng genomic DNA (Roche Applied Science, Karlsruhe, Germany), 400 nM sense and antisense primers, 2.5 U Expand 20 kb^{PLUS} polymerase (Roche Applied Science) and 500 nM dNTPs in a final volume of 25 µl. The conditions for PCR were: 92 °C for 2 min, then 10 cycles of 92 °C for 10 s, 62 °C for 30 s and 68 °C for 18 min, followed by 20 cycles of 92 °C for 10 s, 62 °C for 30 s and 68 °C for 18 min with 10 s per cycle increase, and a final extension at 68 °C for 7 min. The PCR products were cloned into pCR-TOPO XL-1 (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. Inserts were sequenced by the dideoxy chain termination method using an automated DNA sequencer.

Southern blotting of genomic DNA

Genomic DNA was purified from human placenta by a standard procedure using the Nucleobond Ax Kit (Macherey-Nagel, Düren, Germany). Twenty micrograms of genomic DNA were digested with the restriction enzymes SstI, XbaI, BglI or BamHI, separated by electrophoresis on a 0.9% agarose gel and blotted onto positively charged nylon membrane. Genomic fragments were mapped by hybridisation with a digoxigenin-labelled probe generated by PCR with primer pair PGDH-8/PGDH-36 (see Table 1). The blot was hybridised overnight at 68 °C in 0.25 M Na₂HPO₄ (pH 7.2), 20% SDS, 1 mM EDTA and 0.5% blocking reagent and then washed and immunodetected following the recommendations of the DIG

Table 1 Sequence of human PGDH-specific oligonucleotide primers. Positions on the human PGDH cDNA are given relative to the ATG start codon

Primer name	Sequence	Position (orientation)	Application
PGDH-8	CCAGCAGTGGCTGCACCATG	–17–+3 (s)	Southern hybridization
PGDH-36	TGCTCATGCAGGGCAGCTTTAC	234–266 (as)	
PGDH-37	CAGTGCATGTGGCTGACCAGCAACAAC	184–212 (s)	Amplification of intron 2
PGDH-38	ACCAAAATGTCCAGTCTTCCAAAGTGGTCTACA	234–266 (as)	
PGDH-39	TGCTGGAGTGAATAATGAGAAAACTGGGAAAAA	273–306 (s)	Amplification of intron 3
PGDH-40	TGTAATCCAAACCAAGATAGGTTCCACTGATAACA	330–364 (as)	
PGDH-41	AAGGCGGCATCATTATCAATATGTCATCTTTAGCA	386–420 (s)	Amplification of intron 4
PGDH-42	ATCCAACATATGCCATGCTTTGAAGCACAATAAACC	447–481 (as)	
PGDH-43	CTTCAAAGCATGGCATAGTTGGATTACAC	458–487 (s)	Amplification of intron 5
PGDH-44	AATGGCATTTCAGTCTCACACCACTGTTTATA	513–543 (as)	
PGDH-45	CAGCCATCCTTGAATCAATTGAAAAAGAAG	563–592 (s)	Amplification of intron 6
PGDH-46	AATGAGTGTTATCAATCCATTGGCAATCAA	670–699 (as)	

(s), sense; (as), antisense.

Luminescent Detection Kit protocol (Roche Applied Science). The chemiluminescence substrate CDP-Star (Tropix, Bedford, MA, USA) was used for visualisation following the manufacturer's protocol. Light emission was recorded on Kodak XAR-5 film.

Cell culture

Cell lines used in this study included the human choriocarcinoma cell line JEG-3 and the Jurkat human leukaemic T lymphoblast cell line (European Collection of Animal Cell Cultures, Salisbury, Hants, UK). JEG-3 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 with 10% fetal calf serum (FCS), and Jurkat cells were maintained in Iscove's minimal essential medium with 10% FCS. All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin.

Reporter gene constructs and expression vectors

All luciferase reporter gene constructs were generated in the pGL3-Basic plasmid (Promega, Mannheim, Germany). The PGDH promoter/luciferase reporter constructs PGDH-2368/luc3 and PGDH-388/luc3 have been described pre-

viously (Greenland *et al.* 2000). For construction of the minimal promoter vector PGDH-80/luc3, the fragment –80/+8 relative to the ATG start codon was created by annealing complementary oligonucleotides with BglIII and HindIII overhangs and insertion into the respective sites of pGL3-Basic. The PGDH-DE/-80/luc3 vector was constructed by generating a –2152/+1944 fragment (DE, distal element) by PCR, using PGDH-2368/luc3 as the template and primers which added a 5'-KpnI and a 3'-NheI site. This fragment was inserted into the KpnI and NheI sites of the minimal promoter construct PGDH-80/luc3. Individual or combined mutations of the AP-1 and Ets binding sites were introduced into PGDH-388/luc3 by site-directed mutagenesis (Quik Change PCR Mutagenesis System; Stratagene, Amsterdam, The Netherlands). For generation of PGDH-388 Etsmut/luc3, the nucleotide G (–220) was mutated to A using complementary oligonucleotides (5'-CGGTCTGCGAGTCCaGAAGGCAAA GATCG-3'; sense sequence) with a point mutation (lower case) to destroy the Ets binding site (underlined). The PGDH-388AP1mut/luc3 construct was generated by using complementary oligonucleotides (5'-CAGGGGGCAGGTGACAtg GTCGTGGGTTCC-3'; sense sequence) with two point mutations (lower case) (–166/–165) to destroy the AP-1 binding site (underlined).

These oligonucleotides were also used to mutate the AP-1 site of PGDH-388Etsmut/luc3 for generation of the double mutant PGDH-388AP1/Etsmut/luc3. The inserts of the PCR products were excised with Acc65I and BglII and inserted into the respective sites of pGL3-Basic. Sequence analysis verified the presence of the mutations.

Site-directed mutagenesis was also used to mutate individual elements in PGDH-DE/-80/luc3 (see Figs 3 and 7). PGDH-DE/CREB1 mut/-80/luc3 was generated with complementary oligonucleotides (5'-TGTCTGAATCCAGTGCTGtgGTCCTTGTGCC-3'; sense sequence) to mutate the most 5' CREB binding sequence in the distal enhancer, whereas the 3' CREB binding sequence was mutated with complementary oligonucleotides 5'-CTATCCTTGACTCATTGCACTTTGtgCTCAATCAATCTTC-3' (sense) to generate PGDH-DE/CREB2mut/-80/luc3. AP-1 or Ets binding sequences were mutated to generate PGDH-DE/AP1mut/-80/luc3 or PGDH-DE/Etsmut/-80/luc3 respectively, with oligonucleotides 5'-CCAGTTGTCACTATCCTTGACTtgTTGCACTTTGACCTC-3' (sense) or 5'-CCACCGCCAAACTGCTTCtTGTTCTTCCAG-3' (sense) respectively. For combined mutation of the AP-1 and 3' CREB sites (construct PGDH-DE/AP1/CREB2 mut/-80/luc3), complementary oligonucleotide 5'-CCAGTTGTCACTATCCTTGACTtgTTGCACTTTGtgCTCAATCAATC-3' (sense) were employed. After sequence verification, the distal enhancer regions from all mutants were excised with KpnI and NheI and inserted into the respective sites of PGDH-80/luc3.

For construction of the expression vector pcDNA/c-jun, carrying the human c-jun cDNA, human genomic DNA was amplified with Expand polymerase (Roche Applied Science) using primers 5'-TCAGGCAGACAGACAGACAGC-3' (sense) and 5'-GTTAACGAAAGCAGGCCAGAAAGA-3' (antisense). The PCR product was digested with Acc65I and NotI, and the resulting fragment spanning the sequence -704/+1213 relative to the start ATG of c-jun mRNA was cloned into pcDNA3.1(+) (Invitrogen). Human c-fos cDNA was amplified by RT-PCR with Pfx polymerase (Life Technologies, Karlsruhe, Germany) from Jurkat cells which had been stimulated with the phorbol ester TPA (5×10^{-8} M) and Ionomycin (1 µg/ml) (Sigma Chemical Co., St Louis, MO, USA) for 12 h. The primers were: 5'-GAGCAACTGAGAAGCCAA

GACTGAG-3' (sense) and 5'-TAAGGAGAAAGAGAAAAGAGACACA-3' (antisense). The PCR product spanning the sequence -140/+1457 relative to the start codon was digested with NotI at position -110 and ligated into the NotI and EcoRV sites of pcDNA3.1(-).

The coding sequences for human FosB, FosB2, Fra-1 and Fra-2 were amplified by PCR using cDNA prepared from MDA-MB231 breast cancer cells as template with the following primers which carried 5'-overhangs with a HindIII site in the forward (f) primer and a BamHI site in the reverse (r) primer (restriction sites underlined):

FosB-f-Hind: 5'-CGGCAAGCTTACCTCAGCGTGGTCACAGGG-3',

FosB-r-Bam: 5'-GCCGGGATCCCTCTCTCCCCCATGTGTTT-3',

Fra1-f-Hind: 5'-CGGCAAGCTTTGTACCCCGCAGAGCCGCCAG-3',

Fra1-r-Bam: 5'-GCCGGGATCCGGCATCTGCAGGGAGTAGGGC-3',

Fra2-f-Hind: 5'-CGGCAAGCTTATCATGTACCAGGATTATCCC-3',

Fra2-r-Bam: 5'-GCCGGGATCCAGGGAGGTGCACTGGGTAC-3'.

With the FosB primers, the full-length coding sequence and the smaller splice variant generating FosB2 (Yen *et al.* 1991) were simultaneously amplified. PCR was performed in 40 cycles with the Expand High Fidelity PCR System (Roche Applied Science) and an annealing temperature of 58 °C in the presence of 5% dimethyl sulphoxide. The amplification products of 1110 bp (FosB), 970 bp (FosB2), 875 bp (Fra-1) and 1020 bp (Fra-2) were treated with BamHI and HindIII, and ligated into the expression vector pcDNA3.1(+) which had been cut by the same restriction enzymes. Wild-type sequences of the cDNA inserts were confirmed by sequencing on both strands. Expression plasmids pcDNA/JunB (coding for human JunB) and pCMV-2/JunD (coding for mouse JunD) (Harrison *et al.* 1995) were provided by Dr M Robinson (Bristol Dental Hospital, Bristol, UK) and Dr P R Dobner (University of Massachusetts Medical School, Worcester, MA, USA) respectively. An expression vector for human Ets-1 (pSG5-hEts1) was provided by Dr B Wasylyk (INSERM, Illkirch, France), and the CREB expression vector pRc/RSV-FLAG-CREB by Dr R Goodman (Vollum Institute, Portland, OR, USA).

Transient transfections

Transfections of JEG-3 cells were performed by the calcium phosphate precipitation method using the ProFection Mammalian Transfection System (Promega). On the day before transfection, cells were plated onto 24-well culture plates at a density of 0.75×10^5 cells/well. Medium was changed 3 h before transfection. Cells were transfected with 0.75 µg reporter construct and 0.2 µg of each expression vector. For normalisation, 0.05 µg β-galactosidase expression vector pCMV/LacZ (kindly provided by Dr G E DiMattia, London Regional Cancer Centre, London, Ontario, Canada) were included. The total amount of DNA was kept constant by addition of promoterless pOGH (human growth hormone reporter) plasmid. Medium was replaced after 20 h. Cells were harvested 24 h later in passive lysis buffer for measurement of luciferase (Promega) and β-galactosidase activities (GalactoLight; Tropix) in a Berthold luminometer (Isernhagen, Germany). All transfections were carried out in triplicate and repeated several times.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides used as probes or competitors for EMSA were: AP-1 consensus binding site (5'-CGCTTGATGACTCAGCCG GAA-3') (sense sequence, core motif underlined), Ets consensus binding site (5'-GATCTCGAGC AGGAAGTTCGA-3') and CRE consensus (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'). Sequences of PGDH promoter elements (DE/CREB1, DE/Ets, DE/AP1, DE/CREB2, PE/Ets, PE/AP1 and mutations thereof (denoted by the suffix 'm') are shown in Fig. 7. Double-stranded oligonucleotides were end-labelled with [γ - 32 P]ATP and T4 polynucleotide kinase (New England Biolabs, Schwalbach, Germany) and purified on MicroSpin G-25 columns (Amersham Pharmacia Biotech, Freiburg, Germany).

Nuclear extracts were prepared by a modification of the method of Schreiber *et al.* (1989) from Jurkat cells which had been stimulated for 12 h with 5×10^{-8} M TPA and 1 µg/ml Ionomycin.

EMSAs were performed by incubating 12.5 µg nuclear protein (unless indicated otherwise) in bandshift buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5%

glycerol) containing $4 \times$ complete protease inhibitor (Roche Applied Science) in the presence of 1 µg poly(dI-dC) for 15 min at 4 °C. Different amounts of unlabelled competitor binding sites or 1 µl antibody for supershift were added for 30 min, followed by a 30-min incubation with 15 000 c.p.m. of 32 P-labelled probes, all on ice. The protein/DNA mixture was then resolved on a 4% polyacrylamide gel in Tris-borate-EDTA buffer for 1.5 h at 220 V. Dried gels were exposed to X-ray film with an intensifying screen overnight at -80 °C. The following antibodies were employed: against Ets-1/Ets-2 (C-275; Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-fos (4; Santa Cruz), FosB (102; Santa Cruz), Fra-1 (R-20; Santa Cruz), Fra-2 (Q-20; Santa Cruz), JunB (210; Santa Cruz), JunD (329; Santa Cruz), p-c-jun (KM-1, phosphospecific; Santa Cruz) and c-jun (Ab-1; Oncogene Research Products, San Diego, CA, USA). According to the supplier this latter antibody does not produce a supershift but rather blocks a gelshift if added to the protein prior to the AP-1 probe.

Results

Cloning of the introns of the human PGDH gene, and determination of the genomic structure

Comparison of the human and mouse PGDH cDNA sequences revealed a high degree of identity between these species (87%) (Ensor *et al.* 1990, Matsuo *et al.* 1996). According to the mouse splicing sites (Matsuo *et al.* 1997) we designed PCR primers specific to the human PGDH cDNA flanking the putative splice sites. In a long extension PCR with human genomic DNA as the template, intervening sequences containing putative introns were amplified. Amplification products (introns 2–6) were cloned, partially sequenced from both ends and subjected to restriction analysis. These clones complement a 4.6 kb fragment previously cloned by us that contains 3.5 kb of the 5'-flanking region of the human PGDH gene, exons 1 and 2 and intron 1 (Greenland *et al.* 2000).

Based on the restriction pattern of the intronic clones we constructed a restriction map of the human PGDH gene and compared it with the recently completed PGDH gene sequence available at GenBank (accession no. AC096751) (Fig. 1).

Figure 1 Restriction map of the human PGDH gene. The PGDH gene is allocated to human chromosome 4 and is approximately 31 kb long. The indicated restriction sites were obtained from GenBank sequences (accession no. AC09751) and confirmed by restriction analysis of intronic clones with the exception of the BglI and BamHI sites marked by asterisks which were identified by restriction analysis and/or genomic Southern hybridisation. The location of the probe used for Southern hybridisation is shown.

Exons 1–7 span 31 kb on chromosome 4 which has recently been sequenced by the human genome project (Genome Sequencing Center, Washington University, School of Medicine, St Louis, MO, USA). Clone RP11–440I14 contains the PGDH gene, which carries the marker stSG4911. In order to analyse the human PGDH in a genomic Southern blot, placental DNA was digested with several restriction enzymes and probed with a PGDH fragment containing exon 1 sequence from position –17 relative to the start codon, intron 1 and 62 bases of exon 2 (see Fig. 1). The hybridisation pattern of the SstI and XbaI fragments of the genomic DNA agreed with the sequences of clone RP11–440I14 whereas the BamHI and BglI fragments did not (Fig. 2). Since the region of the probe does not contain a BglI restriction site, the two BglI bands on the Southern blot point to the fact that the digest of the placental DNA was incomplete. However, this allowed us to locate two additional BglI sites in introns 2 and 3, indicated by asterisks in Fig. 1, which are not present in the GenBank sequence. An additional BamHI site in intron 2 was located by genomic Southern hybridisation (indicated in Fig. 1 by an asterisk) which was not present in the cloned intron 2, indicating a polymorphism. The hybridisation pattern suggests that only one copy of the PGDH gene exists in the human genome (Fig. 2).

All exon–intron junctions follow the GT–AG rule and show high homology to the mouse PGDH sequence (Matsuo *et al.* 1997) (Table 2). However, the sizes of the human PGDH introns are much larger.

Transcriptional regulation of the human PGDH gene

Within 2.4 kb of the human PGDH promoter several potential binding sites for transcription factors including AP-1, Ets, CREB and CCAAT/enhancer-binding protein are found in two clusters (Greenland *et al.* 2000). The distal element PGDH-DE (–2152/–1944) contains an AP-1 site adjacent to an Ets site and flanked by two CREs (denoted CREB1 and CREB2). The proximal element PGDH-PE (–235/–153) carries an Ets and an AP-1 binding site (Fig. 3).

Figure 2 Southern analysis of the human genome for PGDH sequences. Genomic DNA from human placenta was digested with the indicated restriction endonucleases and subjected to Southern hybridisation with the probe indicated in Fig. 1.

Table 2 Exon–intron junctions of the human PGDH gene. Splice positions are given relative to the ATG start codon in the human PGDH cDNA. Exon sequences are shown in capital letters; intron sequences in lower-case letters. Deviations in the corresponding mouse sequence are indicated in italics

	Size (kb)	Splice position	Exon	Intron donor	Intron acceptor	Exon	Species
Intron no.							
1	0.29	93/94	...CCAAG	gtaagccccgg.... ttgtctatag		GTAGC...	Human
	0.2			 ga tt	 t g		Mouse
2	4.07	217/218	...GAGAG	gtaagacagg.... ttataattag		ACACT...	Human
	3.5			 gtt	 c t tg	 T	Mouse
3	9.18	324/325	...ATTTG	gtgagttatc.... tctggattag		GTTTC...	Human
	3.0			 ac c a	 c		Mouse
4	13.07	421/422	...AGCAG	gtaagggcaa.... tctgtttcag		GACTC...	Human
	1.9			 tt	 c g a c	 G	Mouse
5	2.2	498/499	...CAGCG	gtgaggctac.... taattggcag		TTGGC...	Human
	1.1			 a a a	 g	 A	Mouse
6	1.06	663/664	...TTGGA	gtaagtaaaa.... ttatttttag		CCCAC...	Human
	0.76		 AC		 c c	 G	Mouse

For the characterisation of human PGDH transcriptional control, JEG-3 choriocarcinoma cells were transfected with the PGDH promoter/luciferase reporter gene constructs depicted in Fig. 3. Vectors encoding AP-1 protein (c-jun and c-fos) were co-transfected to activate AP-1-dependent transcription in the absence or presence of co-expressed Ets-1. The PGDH-388/luc3 construct, which encompasses the PE region (–235/–153), was not significantly induced by AP-1 or Ets-1 alone or in combination, nor were the mutants of PGDH-388/luc3 in which the putative AP-1 or Ets binding sites are mutated individually or in combination (Fig. 4). In contrast, the PGDH-DE/-80/luc3 construct, in which the DE region (–2152/–1944) is fused to the minimal homologous promoter element –80/–8 relative to the start ATG, displayed much higher basal activity. In addition, the DE region conferred strong responsiveness to AP-1. Transfected Ets-1 did not have any effect on PGDH-DE/-80/luc3. However, mutation of the Ets binding site reduced overall activity of the PGDH-DE construct, while the responsiveness to

AP-1 was retained (see fold inductions, Fig. 4). Mutation of the AP-1 or the CREB2 site diminished the response to AP-1, and the lowest activities were seen with simultaneous mutation of the AP-1 and CREB2 motifs. The residual stimulation seen with constructs in which the AP-1 site is mutated was also observed with the minimal promoter construct PGDH-80/luc3 and is probably due to a cryptic AP-1 motif in the plasmid backbone, as reported by other investigators (Kushner *et al.* 1994). Mutation of the CREB1 site was without effect on basal or AP-1-stimulated promoter activity (Fig. 4).

We then compared different AP-1 proteins for their ability to stimulate the PGDH-DE/-80/luc3 construct (Fig. 5). Expression vectors for Jun proteins (c-jun, JunB or JunD) were combined with expression vectors for Fos proteins (c-fos, FosB, FosB2, Fra-1 or Fra-2). All these combinations induced the reporter gene more strongly than did any of the vectors alone. Highest reporter gene activity was obtained with c-jun/FosB; c-jun/c-fos yielded an intermediate extent of stimulation comparable with that seen with most of

Figure 3 Map of the human PGDH promoter and reporter gene constructs. The 5'-flanking sequence of the human PGDH gene extending to -2368 bp contains a TATA box and putative binding sites for CREB, Ets and AP-1. DE indicates a cluster of distal elements (co-ordinates -2152/-1944) and PE a proximal element (co-ordinates -235/-153). In the promoter/reporter gene construct PGDH-388/luc3, the putative Ets and/or AP-1 binding sites in PE were mutated as indicated. A fragment encompassing DE was inserted in front of a minimal promoter element (-80/-8 relative to the ATG start codon) and the luciferase gene (luc) to yield reporter gene construct PGDH-DE/-80/luc3. Mutations of individual binding sites were introduced as indicated here; sequences are shown in Fig. 7.

the combinations. No significant induction was obtained on the PGDH-388/luc3 construct irrespective of the co-transfected AP-1 family members (data not shown).

To elucidate the role of the CREB1 and CREB2 sites in PGDH-DE, CREB expression vector alone or in combination with c-jun/c-fos was co-transfected with wild-type or mutated PGDH-DE/-80/luc3 reporter gene constructs (Fig. 6). CREB

alone was able to induce PGDH-DE/-80/luc3, albeit to a lesser extent than was c-jun/c-fos. Co-expression of both families of transcription factors resulted in an additive effect. Mutation of either the AP-1 or the CREB2 site reduced the AP-1 induction to the induction seen with CREB alone. In contrast, mutation of the CREB1 site blunted the response to CREB while still permitting induction by AP-1.

Figure 4 Response of the PGDH promoter to AP-1 and Ets. JEG-3 cells were transiently transfected with the indicated PGDH promoter/luciferase reporter constructs (see also Fig. 3) and expression vectors for AP-1 protein (c-jun/c-fos), Ets-1, a combination thereof (c-jun/c-fos/Ets1), or the empty expression vectors pcDNA3-1 and pSG5 (empty vector). Relative light units of luciferase activities were normalized by β -galactosidase activities. The values depicted in the bar graph are the means $\pm$ S.D. from three independent experiments; the activities are expressed relative to those of the minimal promoter construct PGDH-80/luc3 in the presence of the empty vector. Values shown on the right represent fold inductions where activities obtained for each reporter in the presence of empty vector were set to '1' (means $\pm$ S.D. from three independent experiments).

Characterisation of AP-1, Ets and CREB binding sites in the PGDH promoter by EMSA

In vitro DNA/protein binding studies (EMSA) were used to confirm functionality of potential binding sites in the PGDH promoter. Nuclear extracts from Jurkat cells, which had been stimulated with phorbol ester and Ionomycin, were used as the source of nuclear protein. Jurkat cells express PGDH mRNA at a level comparable with that seen

in placenta (Greenland *et al.* 2000). Oligonucleotides comprising the proximal Ets and AP-1 sequences (PE/Ets, PE/AP1) and oligonucleotides with point mutations of the critical bases (PE/Etsm, PE/AP1m) were used as probes and competitors. Likewise, oligonucleotides carrying the potential binding sites from the distal region (DE/CREB1, DE/Ets, DE/AP1, DE/CREB2) and mutated sequences thereof were employed (Fig. 7).

Figure 5 Response of the PGDH promoter to different AP-1 proteins. JEG-3 cells were transiently transfected with PGDH-DE/-80/luc3 and expression vectors for the indicated AP-1 proteins alone or in combination, or the empty expression vector pcDNA3.1 (empty vector, -). Relative light units (RLU) of luciferase activities were divided by RLU of β-galactosidase activities. The values are the means of triplicate determinations ± S.D. of a representative experiment.

To ascertain the presence of AP-1 proteins in Jurkat nuclear extracts, an AP-1 consensus response element was used as the probe. A pronounced complex was formed which was readily competed by a 20-fold molar excess of unlabelled AP-1 element (Fig. 8). A much weaker DNA/protein complex was formed on PE/AP1 which could be competed by a 100-fold excess of

unlabelled PE/AP1 but not PE/AP1m. A 500-fold excess of consensus AP-1 element was required to reduce binding, indicating that the protein(s) interacting with PE/AP1 are distinct from those interacting with AP-1 consensus.

We next applied the same experimental protocol to the distal AP-1 site, DE/AP1. This probe produced a strong complex with Jurkat nuclear extracts, comparable with that obtained with the consensus AP-1 element (Fig. 9A). Binding was competed by 20-fold excesses of unlabelled DE/AP1 and consensus AP-1 elements, but not by the mutated DE/AP1 site (DE/AP1m) even at a 500-fold molar excess. By supershift analysis, proteins binding to DE/AP1 could be identified as c-fos, JunB, JunD and phosphorylated c-jun, whereas antibodies to other members of the AP-1 family (FosB, Fra-1, Fra-2) did not interact (Fig. 9B).

The proximal and distal Ets binding elements were then analysed. Jurkat cells were shown to contain significant amounts of nuclear proteins binding to an Ets consensus site. A 100- to 500-fold excess of unlabelled Ets consensus was required to compete this binding (Fig. 10A and B, lanes 1–4). PE/Ets also strongly bound proteins from Jurkat cells, and competition was achieved with 20- to 100-fold excess of unlabelled PE/Ets but not with PE/Etsm. However, consensus Ets element even at a 500-fold excess did not compete for this binding (Fig. 10A, lanes 9–14). An antibody to Ets-1/Ets-2 did not produce a supershift of the complex formed with PE/Ets (Fig. 10A, lanes 15–16).

The distal Ets site, DE/Ets, also readily formed a complex with Jurkat nuclear proteins which could be competed by 100- to 500-fold excess of unlabelled DE/Ets but not mutated DE/Etsm. However, in contrast to PE/Ets, binding to DE/Ets could also be competed by Ets consensus element at a 100-fold excess (Fig. 10B, lanes 5–14). Again, an Ets-1/Ets-2-specific antibody did not supershift the complex formed on DE/Ets (Fig. 10B, lanes 15–16), indicating that an Ets factor other than Ets-1 or Ets-2 binds this motif.

Finally, the potential CREB binding sites in the distal PGDH promoter region were subjected to gelshift analysis. Jurkat nuclear extracts contained significant amounts of CRE binding proteins, as shown in Fig. 11 (A, B and C, lanes 1–4) on a CRE consensus probe. Competition was achieved with 20- to 100-fold excess of unlabelled CRE. The 5'

Figure 6 Response of the PGDH promoter to CREB. JEG-3 cells were transiently transfected with the indicated PGDH promoter/luciferase reporter constructs (see also Fig. 3) and expression vectors for CREB or c-jun/c-fos alone or in combination, or the empty expression vector pcDNA3.1 (empty vector). Relative light units of luciferase activities were normalized by β -galactosidase activities. The values depicted in the bar graph are the means $\pm$ S.D. from three independent experiments; the activities are expressed relative to those of the minimal promoter construct PGDH-80/luc3 in the presence of the empty vector. Values shown on the right represent fold inductions where activities obtained for each reporter in the presence of the empty vector were set to '1' (means $\pm$ S.D. from three independent experiments).

CREB element of the PGDH promoter, DE/CREB1, also formed a complex with nuclear extracts which was competed both by unlabelled DE/CREB1 and CRE consensus at a 100-fold excess, but not by the mutated sequence, DE/CREB1m (Fig. 11A, lanes 5–14). Different binding characteristics were observed with the 3' CREB element, DE/CREB2. The pronounced complex formed with nuclear proteins from Jurkat cells was readily competed by 20-fold excess of unlabelled DE/CREB2, but not by DE/CREB2m or by consensus CRE even at 500-fold molar excess (Fig. 11B, lanes 5–14). The latter observation indicated that proteins binding to DE/CREB2

were distinct from CRE binding proteins such as CREB or CRE modulator (CREM). We therefore tested whether DE/CREB2 possibly interacted with proteins from the AP-1 family of transcription factors. To that end, CRE consensus, DE/CREB1 and DE/CREB2 were prepared as probes and incubated with Jurkat nuclear extracts. For competition, AP-1 consensus element was employed. All three probes formed complexes with the nuclear proteins. These were fully competed by as little as 20-fold excess of AP-1 consensus in the case of CRE and DE/CREB1, but not in the case of DE/CREB2. Binding to this probe could not even be competed by a 500-fold excess of AP-1 sequence

Figure 7 Sequences of potential binding sites and mutations thereof in the PGDH promoter. (A) Ets and AP-1 binding elements in the PGDH-PE region (co-ordinates -235/-153 relative to the start ATG) are underlined and bases predicted to be critical for binding are shown in bold type. The sense sequences of double-stranded oligonucleotides used as probes or competitors in EMSA are shown below the genomic sequence; mutated oligonucleotides carry the suffix 'm', and mutated bases are in bold lower case letters. (B) A portion of the PGDH-DE region, encompassing two potential CREB binding sites (CREB1, CREB2), an Ets and an AP-1 binding sequence is shown. Note that the Ets binding sequence is on the lower strand, and the corresponding oligonucleotides are printed in the 3'-5' direction.

(Fig. 11C). Taken together, proteins binding to DE/CREB2 cannot be competed by AP-1 or by CRE consensus elements, whereas DE/CREB1 appears to bind both AP-1 and CREB proteins with similar affinities.

Discussion

The complete human PGDH gene including 3.5 kb of the 5'-flanking region has been cloned. Alignment of splice junctions with the mouse gene shows a high degree of conservation but the sizes of the human PGDH gene introns are much larger. While the human gene spans 31 kb, the mouse PGDH gene is only 11.3 kb in size (Matsuo *et al.* 1997). Within 2.4 kb of the 5'-flanking region of the human PGDH gene, five putative AP-1, four Ets and two CREB transcription factor binding sites have been identified by computerised search (Greenland *et al.* 2000). In a previous study, using the full-length promoter construct PGDH-2368/luc3 transfected into primary cultures of myometrial smooth muscle cells, we observed induction of transcription in response to phorbol ester.

Figure 8 Gelshift analysis of the proximal AP-1 binding sequence. Jurkat cell nuclear extract (5 µg) was incubated with ³²P-labelled double-stranded AP-1 consensus element (lanes 1-4) or the PE/AP1 element from the PGDH-PE region (see Fig. 7A) (lanes 5-14). Competition (Comp.) was carried out with 20-, 100- or 500-fold molar excess of unlabelled AP-1 consensus element (lanes 2-4, 12-14), PE/AP1 element (lanes 6-8) or a mutation thereof (PE/AP1m; lanes 9-11). The specific AP-1 complex is indicated by an arrow.

Figure 9 Gelshift analysis of the distal AP-1 binding sequence. (A) Jurkat cell nuclear extract was incubated with ^{32}P -labelled double-stranded AP-1 consensus element (lanes 1–4) or the DE/AP1 element from the PGDH-DE region (see Fig. 7B) (lanes 5–14). Competition (Comp.) was carried out with 20-, 100- or 500-fold molar excess of unlabelled AP-1 consensus element (lanes 2–4, 12–14), DE/AP1 element (lanes 6–8) or a mutation thereof (DE/AP1m; lanes 9–11). The specific AP-1 complex is indicated by an arrow. (B) Jurkat cell nuclear extract was incubated with DE/AP1 as the probe and subjected to supershift analysis by addition of the indicated antibodies to AP-1 proteins (lanes 1–9). A 500-fold excess of unlabelled probe was added in lane 10. Supershifted complexes are indicated by arrows.

This induction was abolished by a dominant negative to AP-1, indicating involvement of activated AP-1 (Greenland *et al.* 2000). In addition, promoter activity in these cells could also be stimulated by Ets-1, Ets-2 and polyomavirus enhancer activator 3 (PEA3), members of the Ets family of transcription factors, and Ets-dependent activation was repressed by co-expression of a dominant negative to AP-1, pointing to a co-operation between Ets and AP-1 proteins. Two regions in which putative Ets and AP-1 sites appear in clusters, PGDH-DE and PGDH-PE, have been analysed more closely in this report.

In transfection experiments we demonstrated that PGDH-DE confers AP-1 inducibility on the minimal homologous promoter in the choriocarcinoma cell line JEG-3, whereas the promoter-proximal region failed to convey significant activation by AP-1, in keeping with the observed strong binding of AP-1 proteins to PGDH-DE in EMSA, but the very weak interaction of AP-1 proteins with PGDH-PE. The core sequence of DE/AP1 represents the high affinity AP-1 consensus TGACTCA (Karin *et al.* 1997, Chinenov & Kerppola 2001), whereas PE/AP1 is not a perfect palindrome (TGACACA). Supershift analysis revealed the presence of c-fos, JunB, JunD and phosphorylated c-jun in DNA/protein adducts formed with DE/AP1 and Jurkat cell nuclear extracts. In co-transfection experiments, most combinations of Jun and Fos proteins were able to activate PGDH-DE, c-jun/FosB being the most potent heterodimer. Surprisingly, even FosB2 enhanced the transcriptional activation by Jun proteins. In the original report, FosB2, which is a C-terminally truncated splice variant of FosB, has been characterised as a negative regulator of transcription (Yen *et al.* 1991).

In JEG-3 cells, Ets-1 alone neither transactivated through the distal nor through the proximal element in transient transfections even though the distal element, DE/Ets, apparently binds Ets factors *in vitro*. Factors present in Jurkat nuclear extracts, which interact with DE/Ets, appear not to be Ets-1 or Ets-2, as evidenced by supershift analysis. The Ets family members specifically binding to DE/Ets remain to be identified. Although the pronounced binding of proteins to PE/Ets could be abolished by a single point mutation in the predicted Ets binding core motif, binding was not effectively competed by addition of

Figure 10 Gelshift analysis of the proximal and distal Ets binding sequences. (A) Jurkat cell nuclear extract was incubated with ³²P-labelled double-stranded Ets consensus element (lanes 1–4) or the PE/Ets element from the PGDH-PE region (see Fig. 7A) (lanes 5–17). Competition (Comp.) was carried out with 20-, 100- or 500-fold molar excess of unlabelled Ets consensus element (lanes 2–4, 12–14), PE/Ets element (lanes 6–8, 17) or a mutation thereof (PE/Etsm; lanes 9–11). For supershift analysis, an antibody to Ets-1/Ets-2 was added (lane 16). The specific Ets complex is indicated by an arrow. (B) The same protocol as in (A) was employed but using the distal Ets element DE/Ets as the probe and DE/Ets and DE/Etsm as competitors (see Fig. 7B).

an excess of consensus Ets binding sequence. We conclude that the factors interacting with PE/Ets are distinct from those interacting with DE/Ets, where binding could be readily competed with

consensus Ets binding site. Even though transfection of the Ets-1 expression vector did not induce reporter gene activity through the distal element in the construct PGDH-DE/-80/luc3, point mutation

Figure 11 Gelshift analysis of the distal CREB1 and CREB2 binding sequences. (A) Jurkat cell nuclear extract was incubated with 32 P-labelled double-stranded CRE (a consensus CREB binding sequence) (lanes 1–4) or the DE/CREB1 element from the PGDH-DE region as the probe (see Fig. 7B) (lanes 5–14). Competition (Comp.) was carried out with 20-, 100- or 500-fold molar excess of unlabelled CRE consensus element (lanes 2–4, 12–14), DE/CREB1 element (lanes 6–8) or a mutation thereof (DE/CREB1m; lanes 9–11). The specific CRE-binding complexes are indicated by arrows. (B) The same protocol as in (A) was employed but using the second CREB element, DE/CREB2, as the probe and DE/CREB2 and DE/CREB2m as competitors (see Fig. 7B). (C) Consensus CRE (lanes 1–4), DE/CREB1 (lanes 5–8) or DE/CREB2 (lanes 9–12) were used as probes. Binding was competed with 20-, 100- or 500-fold molar excess of AP-1 consensus binding site (lanes 2–4, 6–8, 10–12). Specific complexes are indicated by arrows.

of the predicted Ets site reduced promoter activity. It is conceivable that endogenous Ets factors, present in JEG-3 cells, interact with PGDH-DE and contribute to basal transcription.

We showed previously that activation of the full-length promoter construct PGDH-2368/luc3 by the Ets factor PEA3 is repressed by a dominant negative to CREB (Greenland *et al.* 2000). In the present study, we can now demonstrate binding of CREB or related factors to the DE/CREB1 site *in vitro*. The functional significance of this site was further confirmed by the observation that mutation of CREB1 diminished the CREB-mediated induction of the PGDH-DE/-80/luc3 reporter construct. The CREB2 site rather appears to be involved in mediating AP-1 inducibility. The nuclear proteins binding to this site neither interact with a consensus CRE nor an AP-1 site and remain to be identified.

In summary, we have characterised the distal element of the PGDH promoter as an integrator of activating signals by AP-1, Ets and CREB transcription factors.

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