Functional characterization of the human phosphodiesterase 7A1 promoter

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In this paper, the human phosphodiesterase *7A1* (h*PDE7A1*) promoter region was identified and functionally characterized. Transient transfection experiments indicated that a 2.9 kb fragment of the h*PDE7A1* 5′-flanking region, to position −2907, has strong promoter activity in Jurkat T-cells. Deletion analysis showed that the proximal region, up to position −988, contains major *cis*-regulatory elements of the h*PDE7A1* promoter. This minimal promoter region contains a regulatory CpG island which is essential for promoter activity. The CpG island contains three potential cAMP-response-element-binding protein (CREB)-binding sites that, as judged by *in vivo* dimethyl sulphate (DMS) footprinting, are occupied in Jurkat T-cells. Moreover, overexpression of CREB results in increased promoter activity, but, on the other hand, promoter activity decreases when a dominant-negative form of CREB (KCREB) is over-expressed. *In vivo* DMS footprinting strongly indicates that other transcription factors, such Ets-2, nuclear factor of activated T-cells 1 (NFAT-1) and nuclear factor κB (NF-κB), might also contribute to the regulation of h*PDE7A1* promoter. Finally, h*PDE7A1* promoter was found to be induced by treatment with PMA, but not by treatment with dibutyryl cAMP or forskolin. These results provide insights into the factors and mechanisms that regulate expression of the h*PDE7A* gene.

Key words: cAMP, cAMP-response-element-binding protein (CREB), CpG island, phorbol ester, transcription.

INTRODUCTION

Signalling by cyclic nucleotides is a major signal-transduction pathway in eukaryotes. A remarkable aspect of this signalling pathway is the large number of closely related proteins acting at each step of the transduction cascade. This is particularly so at the level of the cyclic nucleotide phosphodiesterases (PDEs), the enzymes involved in the hydrolysis of cAMP and/or cGMP (for reviews see [1,2]). Mammalian PDEs are encoded by at least 19 different genes that, through alternative splicing and differential transcription regulation, give rise to many more variants (> 50). The various isoforms and variants differ in their enzymic properties, cellular localization and sensitivity to signals. The large number of PDEs probably reflects that a precise regulation of the intracellular levels of cyclic nucleotides is of critical importance for multiple cellular processes. The specific physiological roles played by the different PDEs are, however, largely unknown. There is abundant experimental evidence indicating that expression of PDEs is regulated during development and differentiation [1]. The contribution of alternative splicing to the generation of variants has been documented extensively, but, on the other hand, very little is known about the regulation of PDE expression at the transcriptional level. The complex patterns of expression of the PDE genes strongly suggest that a number of developmental, hormonal, signal-induced and tissue-specific factors must be involved in regulating transcription of the PDE genes. However, most of the actual transcription factors and regulatory circuits controlling expression of the PDE genes are still unknown, as identification of the corresponding promoter regions has been reported for only very few PDE genes (*PDE3B*, *PDE4D*, *PDE5A* and *PDE6A*) [3–7].

In this paper, the identification and characterization of the human *PDE7A1* (h*PDE7A1*) promoter region is reported. Three hPDE7A variants have been described (hPDE7A1, hPDE7A2 and hPDE7A3) that are differentially expressed in some tissues [8–11]. hPDE7A1 is highly expressed in tissues of the immune system as well as in several T- and B-cell lines [8,11]. Moreover, in human T-cells, expression of hPDE7A1 is induced following CD3/CD28 co-stimulation and is required for T-cell activation [12]. Similarly, hPDE7A3 is also up-regulated upon T-cell activation [9]. On the other hand, in the adult, expression of hPDE7A2 appears mostly restricted to skeletal muscle and heart [8,10,11]. In this paper we show that a DNA fragment containing 2.9 kb of the 5′ region flanking the transcription start site of hPDE7A1 has strong promoter activity in transient-transfection experiments performed in Jurkat T-cells. Deletion analysis indicates that the proximal region, up to position −988, retains most of the promoter activity. This minimal promoter region has all the characteristic features of a functional CpG island, is induced by treatment with PMA and contains three functional cAMP-response-element-binding protein (CREB)-binding sites. The CpG island also contains binding sites for other transcription factors [Ets-2, nuclear factor of activated T-cells 1 (NFAT-1) and nuclear factor κB (NF-κB)] that, as judged by *in vivo* dimethylsulphate (DMS) footprinting, are likely to participate in the regulation of h*PDE7A1* transcription.

EXPERIMENTAL

Cloning and sequencing of the h*PDE7A1* 5′-flanking region

To clone the h*PDE7A1* 5′-flanking region, a human BAC library (Genome System, St. Louis, MO, U.S.A.) was screened with a ^{32}P-labelled specific probe spanning nucleotide positions 69–796 of the hPDE7A1 cDNA (GenBank® accession no. L12052). Two positive clones, carrying 150–200-kb-long inserts, were obtained. One clone, which contained exon 1, was selected and used to obtain a shotgun library of 3-kb inserts (GATC-Biotech AG, Konstanz, Germany). Screening of the shotgun library was

Abbreviations used: CRE, cAMP-response element; CREB, cAMP-response-element-binding protein; NFAT-1, nuclear factor of activated T-cells 1; NF-κB, nuclear factor κB; DMS, dimethyl sulphate; PDE, phosphodiesterase; h, human; RACE, rapid amplification of cDNA ends; db-cAMP, dibutyryl cAMP; PKA, protein kinase A; PKC, protein kinase C.

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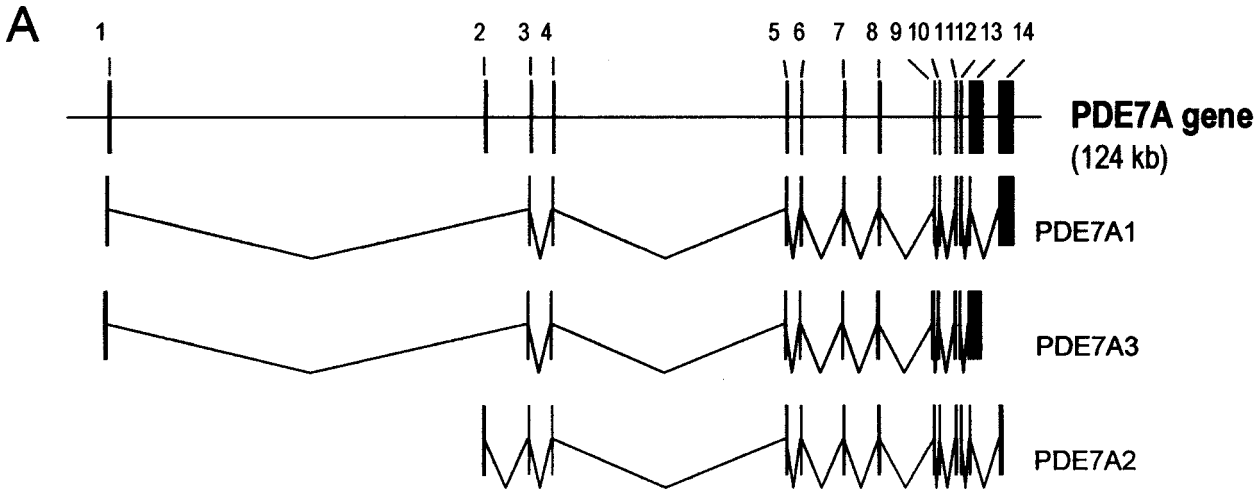

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      SpeI
-2908 ACTAGTCTAGATGTTACTATGAAGATATTTGTGGGTGTGACTACCATTTATAATCCATTGGTTTTAAGCAGATTACTCTCCATGGAGTGGGTGGACCTCA
-2808 TCCAAAGAGTTGAAAGCATTAAGAGCAAAGACTTAGGTTTCCTGAGGATGACTCAGGTCTGCATCAAGACTCCAATGTAGAAACCCTGTGTGAGTTTTCA
-2708 GCCTGTGGTTCTGCTGTGCAGATTTCAGACTCAAGACTGCAATTTCAACTCTTACTTGAATTTCCAGCCTGCTGGCCTGCCCTACAGATTTCAGACTTGC
                Ets-2
-2608 CAGCCTTCACAAAAACATAAGCCATTCCTTAAAATAACTCTCTCCCCCTCTCTCTATATGTACATCCTATGGGTTGTGTTTCTCTGAATAACCTGATTA
                                                NFAT-1
-2508 ATGTGCTGTAAAGAACACTGTAAAGAGCTGAAAAACTCTGGTCTTGAGATACTGTGTGGTATGGTGGAAAACGGCATGGCTCTGAAATCATAGACCAGAGT
                                        GATA3
-2408 TCACTGGGACAAATGACTTATGGTCTTGTAATCTGATTCCTCATCTGTAGAATCAGGATAAAATTACCTGACTTACTTGGCTATTGCGTTGGGTAAAGTT
-2308 AAACATTGTAAGTAGAATGCCTGTCATGGTTCTTGGTTCACAGGTGCACAATAGTAACCAACTATGTTGAAGGGGGATACATTTTTTAGTTATCTACTTA
                        GATA3
-2208 GTGCTCTGAGTGTCATTAAATGATTTAATCAACCTGAGTCTCTTAGATGTTATTAAATGTAATATAGTTATTTTATTTGGCACACAGTAACATCACACCA
-2108 ATATCACCTCCTGGATGGTTAGATTTACACAGAATTCAAACATTATTTGAGGTTAGTTCTTGCCACTTACCTGTGTAAGGTCTAGGGCAAGCGTGTTTCC
                                GATA3   NFAT-1
-2008 ATGAGCCTATTCTCAACTTAGGTACTAATAATTACCTCATGAGGTTTTGTGAGAAGTAAACGTGATAAACGTAGGAAAACTTGCTCAGTATAATCCCAAG
-1908 CATATAGTAGATGCTCAATACATCTACTAGAAGTTGTGGGAGTAATTTGGATTTCAAAGATGAGTTGGATCAGCTGTACTAAAGGGGAGCTTACATCATC
-1808 TAATGAAGACAAAATCCACAAGACCACGTTGGAGGTCATAGTTCTTTTAATAGTAGAAAGCAGACAGGTAGAAAAATATCCACGTAGGAGGTATAAAGTT
                Ets-2   GATA3
-1708 TTAGCAAGAGAAAAGAAACATGCCATGGGAAGATGATAAAAGGTCTACTTTTTGCTCATTTACCGGATTATGTGCATTTGGTATGTGATCTCATCATCAG
-1608 TGGAAGTTATGCAGTGCTTTCAAAAGCCTTCCACCTTGTCAGTGGCTAGTCATCACAAATCAGTAGAAGGGTGGATGCCCTAAGAACTGAACCAAAGCAA
                                                        HindIII
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-1408 GAGAAATGTGACAAAATGAACACTCAAATTACATATCTAGCTTACAAAACAGGTCAAGAGCTCTTTATCTTTTCTCGCTTTCGAAAAATCGAAAGTCAAC
-1308 AAACAACACTTTTGGTAGAGGGGCATGCAGTCTACTTTTTCCCTTTGTCTTCTCAGTATCATCCTTTCTCATTCTTTTCACCCATACTCTCACTTCTTTC
                                                                AP2
-1208 TCAGTCTTGGTTTTTTCCCTTTGTCTCCGGGTCCCCTTCTCACTTCCGAATTCTCGGCTCCCCTCTCCGCCCCTCGGGCTCGGGATGGCCCCGGGGCCGC
                                AP1     CREB                    AP2     CREB    AP4
-1108 ATCTGCCAGGCGAGCGCTGGAGCCTGGCCCCACCGAGGGACTGGCAGTGACGTAGCCAGGAGGACGGTGCTCGCGCGGCCCGCTGACGTCACTGCCGCAG
                        EcoRI           NFAT-1
-1008 CGGTCTCTAGCGAAACCCATGAATGGGAATTCAAGGAGGGAAAAGCCAGCAGTGACCCCGCGGTCAGGGCTGAGGACGGCGACTGCGCCGGGTGCTTTGG
 -908 GTTTCTGGGGTCGGCAGGCCGGAACGCCGCCTCTAGCGCGGGACGAGCACACCCCATAGCCTAGGCGCGCTGGAACGCACGAATTTAGCGCTCGGGGGGG
      NF-kB   Myb     Sp1                             Ets-2
 -808 ATGCCCCGAAACGGAGGGGCCTCGTGCGCACGCGCCTTGGCTGGCGAGGAGCTAGGGAAGGGAGTCACGGCGGGGCGCGAAGGGAACTGCGGATGAGATA
          NFAT-1
 -708 GATGGGGGAAAACGTTTGTCGGGAGGCGACTCCAACAGAGCCCCAGGGTGCAGAACACAAGGAAGGCTACTGTCGCCCTCGGCCGCTTGCCAGTCTCCCT
                Sp1                             AatII CREB/AP1  AP4     C-EBPβ
 -608 GGGCGCTGCGGCGGCGCGGGGCGCTGGGCCTGCGGGCGCGGCGTCGTGACGTCACGGTGGCGGCAGCCGTGGTTGGCGCAAGGTCCGCGGCTGCAAAGTG
                                                                Sp1
 -508 TGAATTACGCCGGGCTCGCGCCGGCGGCGGAGTGAAGTCAGGCTCCGGGGGAGGGGAGGAGCAGGACTCCGGCGGCGGCGGCGGCGGCGGC
                                                AP4
 -408 GGCCCGAGCTGGTCGCTGCTCCTGAGTCCCGCGCGCCCTGGGTCTGGCCGCTGGCTCCAGCTGTCTTTTCTTGCACTGCTTTGCTCCGTTCGTGCTGCGC
                KpnI                    AP2
 -308 TCGAACTCTGCAGTCCGGGTACCTGCTCCTCTCGGCCGAGGAGCCCCAGGGCGGCGGGGTTCCCTTTCCGACTTTGCCTGTGCTGGCCCCGCTTGCACCC
                                                        AP2
 -208 CGTCCCCCCTCGCCTGGAGCGAGGCGCGCGCCGGTCCTGGTAATTGGCGCCGCGGCCGCCGCGGAGCCTTCCCGCCGGCGGCCCGAGCGGAGGGCTGGGG
 -108 TTGGGGAGGCGTGAGCGCGGCTTCCCAGCCCGGGGTCGGTCCCCGCCCGCCCTGCCCCCGTCTCGGCCCGGAGCCCCGAGCCGCGGCCCCCTCCCACTG
      PstI  +1
   -8 CAGGGGCCGGCGGCCGCGGCAGGGCGGGCGCCGCGCGGAGGCAGGGCGGGCGTATTCAATGGAAGTGTGTTACCAGCTGCCGGTACTGCCCCTGGACAGG
                                                        M  E  V  C  Y  Q  L  P  V  L  P  L  D  R
   93 CCGGTCCCCCAGCACGTCCTCAGCCGCCGAGGAGCCATCAGCTTCAGCTCCAGCTCCGCTCTCTTCGGCTGCCCCAATCCCCGGCAGCTCTCTCaggtaa
        P  V  P  Q  H  V  L  S  R  R  G  A  I  S  F  S  S  S  S  S  A  L  F  G  C  P  N  P  R  Q  L  S
  193 Tcgccgccgccgggcccacacttgagggcttctctcttttgtttccctctgcccacccagcttcacctcctctacaaccccagcccgcgcattgtccat
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  493 tctggaagttgagtctttattccttagtcgtggcccagagtcacctgtgctcccccgccttgcaacatacctgtccccttcccatacacacaactgtac
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Figure 1 Genomic organization of the h*PDE7A* locus

(**A**) Schematic representation of the exon/intron structure of the three h*PDE7A* variants (h*PDE7A1*, h*PDE7A2* and h*PDE7A3*). Exons are numbered and shown as vertical bars. (**B**) Nucleotide sequence of the 5′-flanking region of h*PDE7A1* (EMBL accession no. AJ457788). The sequence from position −2908 to +592 is shown. The +1 position corresponds to the mapped transcriptional start site (GenBank accession no. L12052). The position of the ATG codon is marked by an arrow, and sequence translation for the first exon is given. Lower-case letters indicate intron sequences. The positions of some relevant potential binding sites for transcription factors are shown by boxes. Cleavage sites for some restriction enzymes are also indicated.

performed by PCR using primers derived from the sequence of exon 1. Positive clones were then sequenced and new primers were derived from the determined sequence. The process was reiterated to cover the 5′-flanking region up to position −4137.

5′ Rapid amplification of cDNA ends (RACE) experiments

To obtain full-length 5′ ends of hPDE7A1/A3 cDNAs, 5′ RACE experiments were performed from total Jurkat RNA with a Gene Racer™ kit (Invitrogen) in accordance with the manufacturer's instructions. In these experiments, several primers derived from the sequence of the 5′ region common to h*PDE7A1* and h*PDE7A3* (GenBank accession no. AF332652), were used: 5′-GTAACATCCGCAGCGTGG-3′ (nucleotides 712–695), 5′-GGGAATTTGAAACCGCAGTACCAC-3′ (nucleotides 447–424), 5′-GCTGATGGCTCCTCGGCGGCTG-3′ (nucleotides 134–113) and 5′-CATTGAATACGCCCGCCCTGCC-3′ (nucleotides 53–32; sequence co-ordinates are derived from GenBank accession no. L12052). RACE products were cloned into a pGEM-T Easy vector (Promega) and sequenced.

Computer-aided analysis of potential CpG islands

Prediction of putative CpG islands was performed by using CpG finder at the European Bioinformatics Institute (EMBL; http://www.ebi.ac.uk/). The parameters defined were: CpG length > 200 bp, G + C > 50 % and a 'CpG value' (the ratio of observed to expected frequencies of the CpG dinucleotide) of at least 0.6. The resulting putative CpG islands were merged if they were within 100 bp of each other and if the CpG value of the merged island was still higher than 0.6.

Southern-blot analysis

Genomic DNA was obtained from Jurkat T-cells by the proteinase K digestion method. Genomic DNA (30 μg) was digested with *Bam*HI, *Bam*HI + *Hpa*II or *Bam*HI + *Msp*I. Digested DNAs were then analysed by electrophoresis on 0.8 % agarose/Tris/borate/EDTA gels, transferred to nylon and hybridized with a randomly labelled ^{32}P probe spanning nucleotides −1425 to −290 of the h*PDE7A* 5′-flanking region.

Determination of the patterns of DNA methylation

DNA methylation at cytosine residues was determined by the bisulphite-conversion reaction [13]. Genomic DNA from Jurkat T-cells (10 μg) was digested with *Bam*HI + *Pvu*II, denatured by treatment with 0.3 M NaOH for 30 min at 37 °C and incubated at 55 °C for 16 h in 3.1 M sodium bisulphite/0.5 mM hydroquinone. Modified DNAs were amplified by two rounds of PCR using nested primers. PCR products were purified by gel electrophoresis and sequenced.

Determination of promoter activity

Promoter activity was determined by transient-transfection experiments performed in Jurkat T-cells using a luciferase reporter system. For this purpose, a 2.9-kb DNA fragment of the h*PDE7A* 5′-flanking region (positions −6 to −2907) was fused to the promoter-less firefly luciferase gene of plasmid pGL3b (Promega). 5′ and 3′ deletions of this 2.9-kb fragment were obtained by cleavage with appropriate restriction enzymes. The

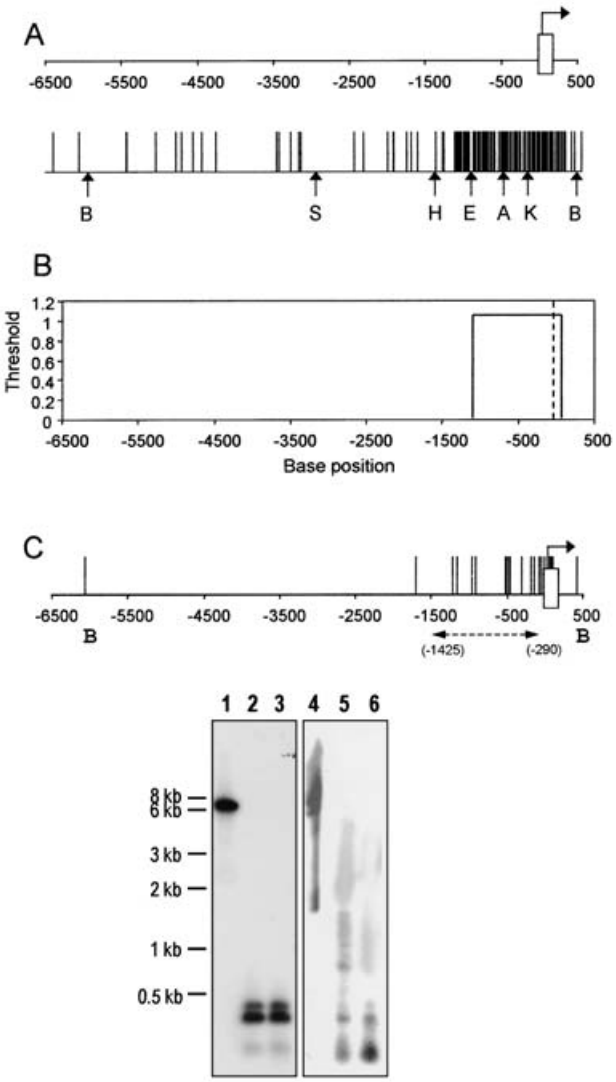

Figure 2 The proximal region of the 5′-flanking region of h*PDE7A1* contains a CpG island

(**A**) Distribution of the CpG dinucleotide through the h*PDE7A1* 5′-flanking region. Vertical lines represent CpG dinucleotides. The box indicates the position of exon 1 and the arrow indicates the position of the ATG codon. Cleavage sites for some restriction enzymes are indicated (A, *Aat*II; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Spe*I). (**B**) Distribution of the CpG value through this region. CpG value, expressed as the ratio of observed to expected frequencies of the CpG dinucleotide, was determined using CpG finder at the European Bioinformatics Institute. Only values higher than 0.9 are represented. The position of the ATG codon is shown by the dotted line. (**C**) Southern-blot analysis of the patterns of cleavage by *Msp*I and *Hpa*II at the h*PDE7A1* 5′-flanking region. The distribution of *Msp*I/*Hpa*II restriction sites through this region is shown schematically. The position of the first exon (box), the ATG codon (arrow) and the two *Bam*HI restriction sites (B) flanking this region are indicated. The probe used in this analysis is also indicated (dotted line). The patterns of cleavage by *Bam*HI (lanes 1 and 4), *Bam*HI + *Hpa*II (lanes 2 and 5) and *Bam*HI + *Msp*I (lanes 3 and 6) of genomic DNA obtained from Jurkat T-cells (lanes 4–6) and of a BAC clone carrying the h*PDE7A* genomic region (lines 1–3) are presented. Numbers on the left indicate appropriate molecular-mass markers.

various promoter constructs assayed are summarized in Figure 3 (see below). For transient-transfection analysis, Jurkat T-cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. Prior to transfection, cells were washed with non-supplemented Dulbecco's modified Eagle's medium, resuspended in serum-free medium

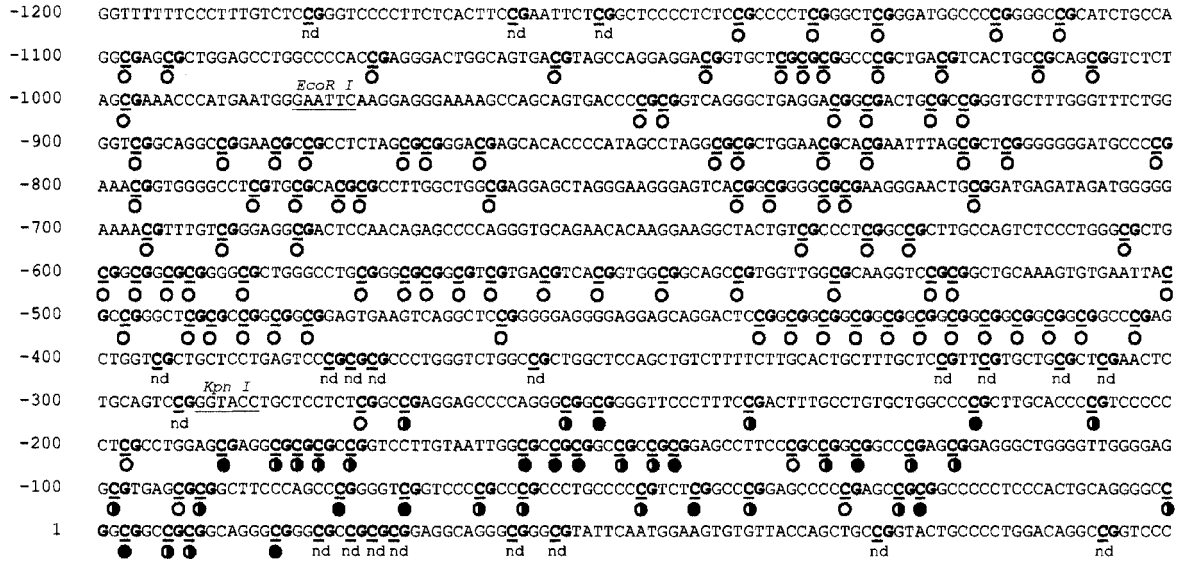

Figure 3 Pattern of CpG methylation through the CpG island (position −1200 to +100) as determined by the bisulphite-conversion reaction

Empty circles are unmethylated cytosines. Half and full circles indicate, respectively, partially methylated and fully methylated residues. nd, not determined.

at a density of 40×10^6 cells/ml and transfected by electroporation (250 V, 950 μF) with 15 μg of the corresponding pGL3b plasmid. To normalize for changes in transfection efficiency, 3 μg of pRL-TK vector, expressing *Renilla* luciferase under the control of the thymidine kinase promoter, was always co-transfected together with the pGL3b plasmids. After electroporation, cells were split into 3-ml aliquots (1×10^6 cells/ml) and grown for 48 h before determination of luciferase activities. Luciferase activities were determined by the dual-luciferase assay system (Promega), according to the manufacturer's instructions. Treatment with PMA or dibutyryl-cAMP (db-cAMP) was performed 24 h after transfection by growing the cells in the presence of 20 nM PMA for 24 h or in 2 mM db-cAMP for 12–24 h before determination of the luciferase activities.

When CREB, KCREB (a dominant-negative mutant form of CREB) or the catalytic subunit of protein kinase A (PKA) was over-expressed, plasmids RSV-CREB, RSV-KCREB and SRα-PKA were used [14,15]. Increasing amounts of the corresponding plasmids were transfected into Jurkat T-cells by electroporation together with 5 μg of either −1.4*PDE7A* or −0.98*PDE7A* promoter constructs. To normalize for changes in transfection efficiency, 1 μg of pRL-TK (SRα-PKA) or CMV-β-gal, expressing LacZ under the control of the cytomegalovirus promoter, RSV-CREB and RSV-KCREB was added to the transfection. Total DNA concentration was adjusted to 20 μg by the addition of pUC19. Cells were harvested 24 h post-transfection and processed as described above.

In vivo DMS footprinting

For *in vivo* DMS footprinting, exponentially growing Jurkat T-cells (10^7–10^8) were harvested, washed twice with PBS and treated with 0.1% DMS for 5 min at room temperature. The reaction was stopped by the addition of 100 mM β-mercaptoethanol in PBS. Cells were then washed with cold PBS and incubated at 50 °C for at least 3 h in 100 mM NaCl, 10 mM Tris/HCl (pH 8), 25 mM EDTA, 0.5% SDS and 0.2 mg/ml proteinase K. When the reaction was performed with naked DNA, 10 μg of purified genomic DNA from Jurkat T-cells was treated with 0.5% DMS for 4 min at room temperature. After DMS treatment, DNAs were purified and cleaved by treatment with 1 M piperidine at 90 °C for 30 min. The patterns of DMS cleavage were then determined by ligation-mediated PCR as described in [16].

RESULTS AND DISCUSSION

The proximal region of the h*PDE7A1* 5′-flanking region contains a CpG island

Figure 1(A) shows the genomic organization of the h*PDE7A* gene which is located at chromosomal position 8q13 [17]. The coding region spans 124 kb and contains 14 exons. The h*PDE7A2* isoform starts at exon 2, which is spliced out in the h*PDE7A1* and h*PDE7A3* isoforms. The current version of the freely accessible Celera (http://public.celera.com/humanpub/index.jsp) sequence of the human genome covers most of the *PDE7A* locus (genomic scaffold segment GA_x2HTBL474CD:1.500000), but contains a gap of approx. 2.5 kb that includes part of the first intron, the first exon and approx. 1.5 kb of the 5′-flanking sequence. The database of the Public Consortium (http://www.ensembl.org/Homo_sapiens/) contains a larger gap in this region (EMBL accession no. AC055822). This gap was covered by four overlapping clones obtained as described in the Experimental section, and the region from position −5169 to +1700 was sequenced (EMBL accession no. AJ457788). Figure 1(B) shows the nucleotide sequence of this region from position −2908 to position +592.

The longest reported hPDE7A1 cDNA (GenBank accession no. L12052) shows an unusually short 5′-untranslated region, only 50 bp long (Figure 1B). This result was confirmed in 5′ RACE experiments using different oligonucleotides derived from the sequence of the 5′ region of the hPDE7A1 cDNA (see the Experimental section for a detailed description of the primers used). Consistent with these results, the 5′ region immediately flanking the mapped +1 position of h*PDE7A1* shares several features which strongly suggest that it corresponds to a *cis*-regulatory region. On one hand, it contains potential binding sites for several transcription factors (Figure 1B) that are likely

to participate in regulating the expression of h*PDE7A1* (see below). Secondly, although the most proximal region carries no identifiable TATA-box sequence, it shows all of the characteristic features of a functional CpG island (Figure 2) [18,19]. The distribution of the CpG dinucleotide through this region clusters at around position +1 (Figure 2A). This region extends for approx. 1.2–1.3 kb, shows a high C + G content (70%) and has a CpG dinucleotide frequency that is 90% higher than that expected on the basis of its C + G content (Figure 2B), indicating a very high CpG island potential [20]. In mammals, DNA methylation occurs almost exclusively at CpG dinucleotides. A remarkable feature of CpG islands is that they are refractory to DNA methylation [18,19]. In contrast, outside of the islands, CpG dinucleotides are under-represented and mostly methylated. The extent of CpG methylation through this region was analysed by determining its sensitivity to cleavage by *Msp*I, which is insensitive to CpG methylation, in comparison with cleavage by *Hpa*II, which is sensitive to DNA methylation. As shown in Figure 2(C), in genomic DNA obtained from Jurkat T-cells, the pattern of *Msp*I cleavage through this region was very similar to that obtained with *Hpa*II (Figure 2C, compare lanes 5 and 6), as well as to those obtained from a BAC clone containing the h*PDE7A* genomic region (Figure 2C, lanes 2 and 3). The pattern of digestion by *Hpa*II shows, however, the presence of bands of higher molecular mass (Figure 2C, lane 5), which most probably originate from CpG methylation at the regions surrounding the island. These results were corroborated when the actual pattern of CpG methylation through this region was determined by the bisulphite-conversion reaction (Figure 3) [13]. In denatured DNA, unmethylated cytosine residues are converted into thymine by the action of sodium bisulphite. Methylated cytosine is, however, unreactive to sodium bisulphite. As shown in Figure 3, cytosine-to-thymine conversion is observed at most CpG dinucleotides lying within the putative CpG island, indicating that they are unmethylated. Altogether, these results indicate that the h*PDE7A1* 5′-flanking region contains a regulatory CpG island.

In Jurkat T-cells, a 2.9-kb fragment of the h*PDE7A1* 5′-flanking region has strong promoter activity to which the CpG island shows an essential contribution

The actual promoter activity of the h*PDE7A1* 5′-flanking region described in Figure 1 was determined in transient transfection experiments performed in Jurkat T-cells (Figure 4). In these experiments, different DNA fragments from this region were cloned into pGL3b plasmid, fused to a luciferase reporter gene. As shown in Figure 4(A), a DNA fragment extending up to position −2907 shows strong promoter activity. The luciferase activity obtained with the −2.9*PDE7A* construct was 30-fold higher than that obtained with the promoter-less pGL3b vector carrying no cloned DNA fragment. A 1.5 kb deletion of the 5′ region of this fragment, to position −1425, reduced promoter activity to approx. 60% (Figure 4A, −1.4*PDE7A* construct). A larger 5′ deletion, to position −988, showed no further effect on promoter activity (Figure 4A, −0.98*PDE7A* construct). On the other hand, the distal 5′ region, from −2900 to −1116, showed no promoter activity at all by itself (Figure 4A, −2.9*PDE7A*$\Delta_{1.11}$ construct). These results show that the proximal region, up to position −988, contains major *cis*-regulatory elements of the h*PDE7A1* promoter. Actually, the CpG island described in Figure 2 is contained within this region, suggesting an essential contribution of this element to the activity of the h*PDE7A1* promoter. Consistent with this hypothesis, a 5′ deletion to position −560, eliminating the 5′ part of the CpG island, fully abolished promoter activity (Figure 4A, −0.56*PDE7A* construct). Similarly, deletion of the 3′ part of the CpG island also eliminated most of its promoter activity (Figure 4A, −0.98*PDE7A*$\Delta_{0.56}$ construct). These results confirm the fundamental contribution of the CpG island to the regulation of the activity of the h*PDE7A1* promoter. Interestingly, the most proximal 3′ region of the CpG island shows a higher degree of methylation (Figure 3), contains a low number of potential binding sites for transcription factors (Figure 1B) and appears to have a negative contribution to activation, since, for all the constructs tested, deletion of the 3′ region to position −296 results in a slight, though significant, increase in promoter activity (Figure 4B, −2.9*PDE7A*$\Delta_{0.29}$, −1.4*PDE7A*$\Delta_{0.29}$, −0.98*PDE7A*$\Delta_{0.29}$ and −0.56*PDE7A*$\Delta_{0.29}$ constructs).

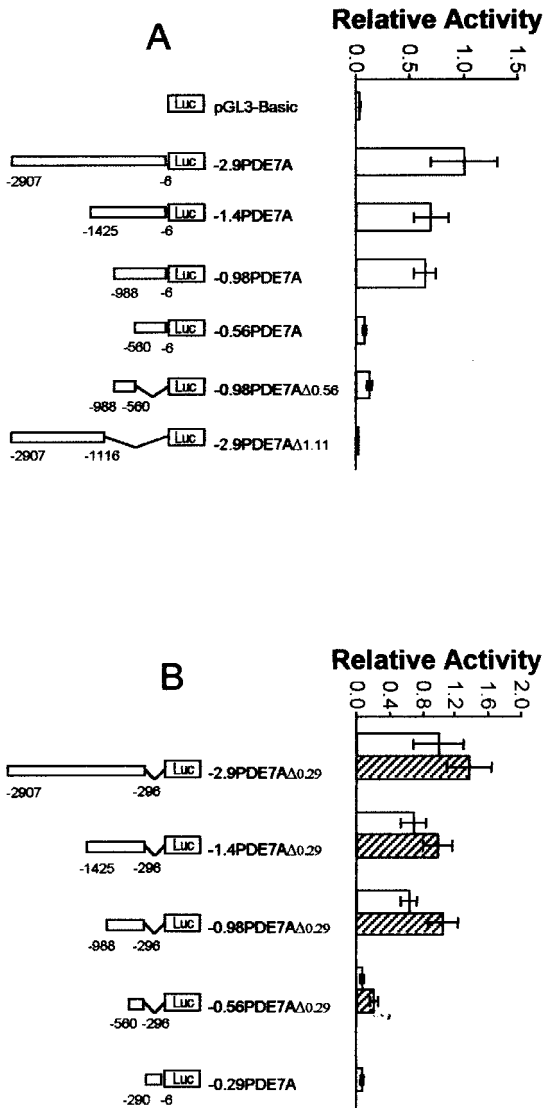

Figure 4 Promoter activity of the h*PDE7A1* 5′-flanking region as determined in transient-transfection assays performed in Jurkat T-cells using a dual-luciferase reporter system

(**A**) Luciferase activities of the promoter constructs indicated on the left are shown. Values are normalized with respect to the activity of the −2.9*PDE7A* construct. (**B**) Luciferase activities of promoter constructs lacking the most proximal region, to position −296 (hatched bars), are compared with those of the corresponding constructs carrying it (white bars). Values are normalized with respect to the activity of the −2.9*PDE7A* construct. Results correspond to means ± S.D. from at least six independent experiments.

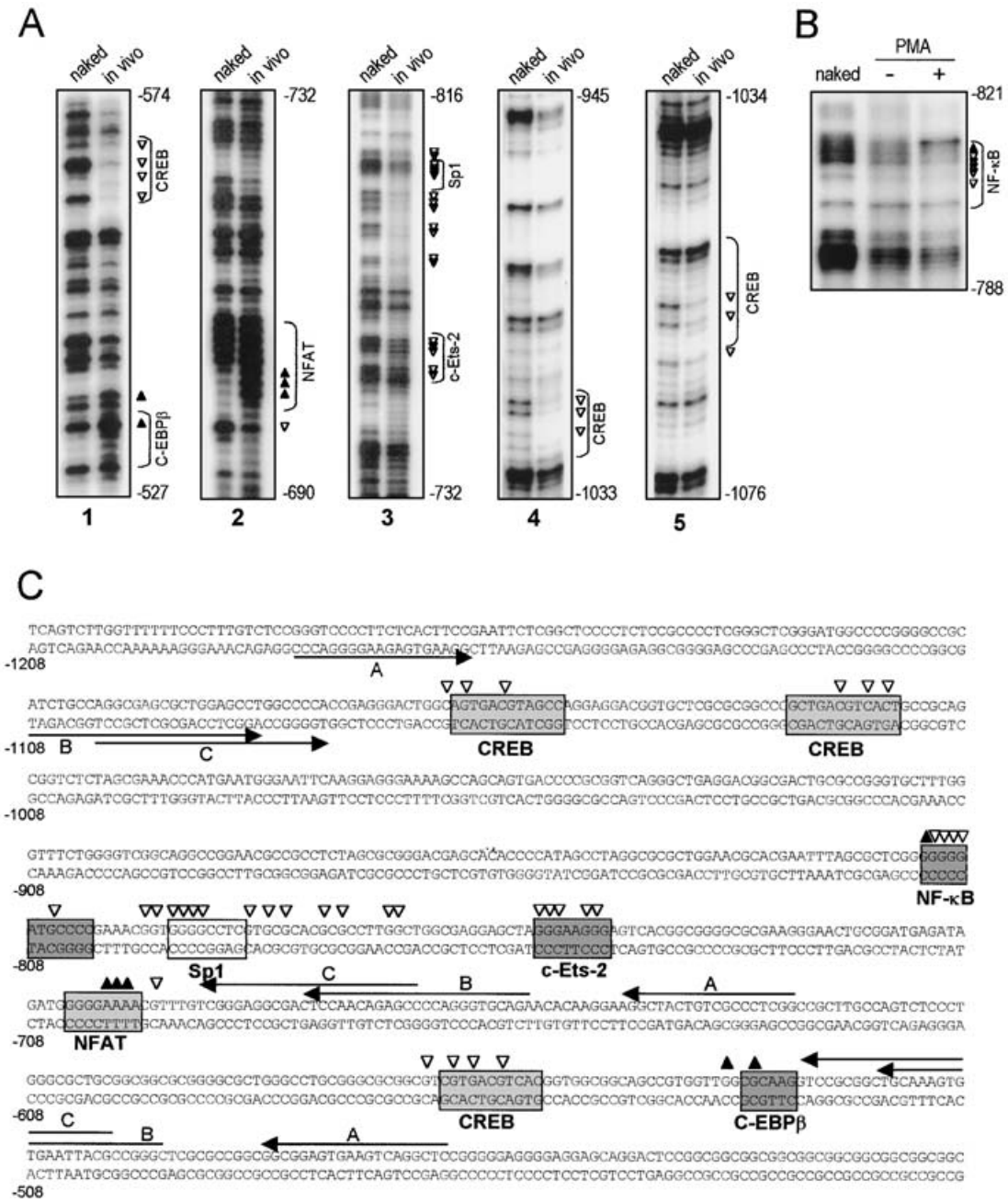

Figure 5 ***In vivo*** **DMS footprinting of h*PDE7A1* promoter**

(**A**) Patterns of DMS reactivity of the h*PDE7A1* promoter regions indicated (lanes 1–5) were obtained in Jurkat T-cells (lanes marked *in vivo*) and in naked genomic DNA (lanes marked naked) as described in the Experimental section. Empty and full triangles indicate hyper- or hypo-reactive residues, respectively. Brackets indicate footprints at binding sites for known transcription factors. (**B**) Patterns of *in vivo* DMS reactivity at the putative NF-κB-binding site occurring at positions −813 to −802 obtained after treatment of the cells with 20 nM PMA (lane +) and in the absence of any stimulation (lane −). The pattern of DMS reactivity of naked genomic DNA is also presented (naked). (**C**) Summary of the results presented in (**A**) and (**B**). Consensus binding sites for transcription factors showing *in vivo* footprints are boxed. Arrows correspond to the sets of primers used to analyse by ligation-mediated PCR the various promoter regions.

h*PDE7A1* promoter contains three potential CREB-binding sites, which are occupied *in vivo*, and is induced by CREB over-expression

The results reported above confirm the promoter activity of the CpG island located at the 5′ region flanking the h*PDE7A1* gene. As mentioned above, this region contains potential binding sites for various transcription factors (Figure 1B) that, characteristic of most functional CpG islands, lie mainly within the 5′ half of the island [18,19,21]. Many of the transcription factors that could potentially bind to this region are known targets of various signalling pathways, suggesting that expression of h*PDE7A1* is tightly regulated. Occupancy of the various potential binding sites for transcription factors was determined in Jurkat T-cells by *in vivo* DMS footprinting (Figure 5). The h*PDE7A1* promoter region contains three cAMP-response elements (CREs; Figure 1B) that

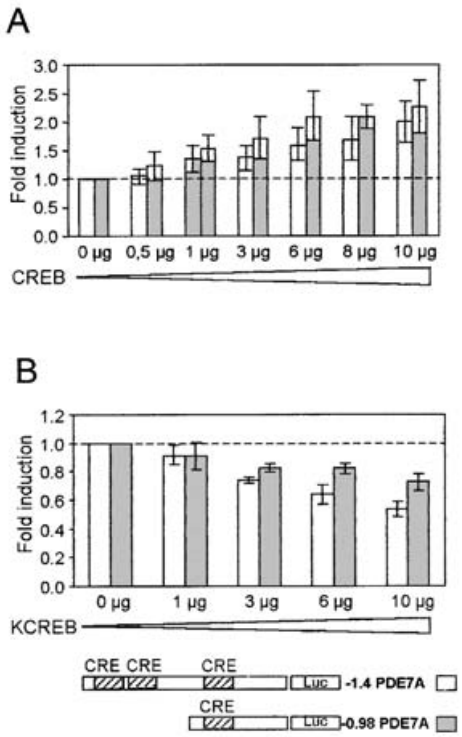

Figure 6 h*PDE7A1* promoter is activated by CREB and repressed by KCREB

Luciferase activities of the indicated promoter constructs were determined after co-transfection with increasing amounts of plasmids expressing CREB (**A**) or KCREB (**B**). Values are normalized with respect to those obtained in the absence of any added over-expression plasmid. Results correspond to means ± S.D. from at least six independent experiments.

are potential binding sites for the CREB family of transcription factors [22,23]. All three CREs show strong DMS footprints *in vivo* (Figure 5A, lanes 1, 4 and 5), indicating that they are actually occupied, most likely by CREB. Some CREB-binding sites, however, share significant homology with binding sites for other transcription factors, such as AP1 or AP2. In fact, in the h*PDE7A1* promoter, the distal and proximal CREs also score as potential AP1 sites, while the intermediate CRE sequence overlaps with a potential AP2 site (Figure 1B). The actual contribution of CREB to the regulation of h*PDE7A1* promoter was addressed by transient expression experiments in which h*PDE7A1* promoter constructs were co-transfected to Jurkat T-cells together with plasmids over-expressing CREB or KCREB, a dominant-negative mutant form of CREB [15]. As shown in Figure 6, the −1.4*PDE7A* construct, which carries all three CREs, is significantly induced by over-expressing CREB (Figure 6A), but is repressed when KCREB is over-expressed (Figure 6B). The −0.98*PDE7A* promoter construct, carrying only the proximal CRE, shows similar induction by CREB and repression by KCREB (Figure 6). Altogether, these results indicate that CREB contributes to the regulation of h*PDE7A1* promoter and strongly argue in favour of the CREs being functional CREB-binding sites.

In addition to the CREs, distinct footprints are also observed at a few other regions of the CpG island. In particular, a strong footprint is observed at position −793 to −768 (Figure 5A, lanes 3) which includes a potential Sp1-binding site (Figure 5C). Similarly, a significant footprint is observed at positions −753 to −747 (Figure 5A, lanes 3), which corresponds to a potential Ets-2-binding site (Figure 5C). On the other hand, a strong hyper-reactivity is observed at the putative NFAT-1-binding site occurring at positions −704 to −697 (Figure 5A, lanes 2). These results suggest that, in addition to CREB, other transcription factors are likely to participate in the regulation of h*PDE7A1* promoter.

h*PDE7A1* promoter is induced by treatment with PMA, but not by treatment with db-cAMP or forskolin

Inducibility by CREB suggests that h*PDE7A1* expression might be regulated in response to changing intracellular levels of cAMP. CREB activity is regulated by phosphorylation, principally by PKA, that is, in turn, the primary target of cAMP stimulation [22,23]. Actually, in B-lymphocytes, h*PDE7A1* expression was found to be up-regulated by increasing the intracellular concentration of cAMP [24]. On the contrary, in T-lymphocytes as well as in Jurkat T-cells, no stimulation of h*PDE7A1* transcription was detected upon treatment of the cells with fenoterol, which increases intracellular levels of cAMP, or 8-bromo-cAMP [25]. Consistent with these results, in our experimental system, no significant induction of the h*PDE7A1* promoter was observed when cells were treated with db-cAMP (Figure 7A) or forskolin (results not shown). Moreover, over-expression of the catalytic subunit of PKA has no effect on the activity of either the −1.4*PDE7A* or −0.98*PDE7A* promoter constructs (Figure 7B). It is possible that, in Jurkat T-cells, h*PDE7A1* promoter is constitutively activated by the intracellular pool of functional CREB. Consistent with this hypothesis, induction of h*PDE7A1* promoter showed no synergism when CREB was over-expressed in cells treated with db-cAMP (results not shown).

On the other hand, a significant increase in the activity of the h*PDE7A1* promoter was observed upon treatment of the cells with PMA (Figure 7C, −2.9*PDE7A* construct). Induction by PMA of the h*PDE7A1* promoter is associated mainly with the CpG island, as shown by the strong induction observed with promoter constructs containing only this promoter element either in full or in part (Figure 7C, −0.98*PDE7A* and −0.56*PDE7A* constructs). The most proximal −296 promoter region is, however, not induced by PMA (Figure 7C, −0.29*PDE7A*) and, also in this case, shows a negative contribution to the activity of the induced promoter. PMA activates protein kinase C (PKC). CREB can also be phosphorylated by PKC, and it was proposed that PKC-dependent phosphorylation affects CREB dimerization and activity [26]. Actually, in lymphocytes, CREB-phosphorylation in response to stimulation of the B-cell surface immunoglobulin was found to be PKC-dependent [27]. Therefore, it would be possible that induction of the h*PDE7A1* promoter by PMA would reflect CREB activation via a PKC-dependent pathway. It is also possible that induction by PKC of the h*PDE7A1* promoter reflects activation of transcription factors other than CREB. Actually, Ets-2 and NFAT-1, which, as judged by DMS footprinting (Figure 5A), are likely to bind the h*PDE7A1* promoter *in vivo*, are known to be activated by PKC [28,29]. Moreover, upon treatment with PMA, a distinct footprint is detected at the putative NF-κB-binding site occurring at positions −813 to −802. In the presence of PMA, the first guanine of the NF-κB-binding site becomes hyper-reactive to DMS, while the rest are protected (Figure 5B). This pattern of DMS reactivity is characteristic of the binding of NF-κB [30,31], which is also activated by PKC. These observations might be relevant in the context of T-cell activation, which involves PKC signalling, and requires induction of hPDE7A [12]. Moreover, the use of selective inhibitors has revealed the general contribution of hPDE7 to the regulation of T-cell functions [32].

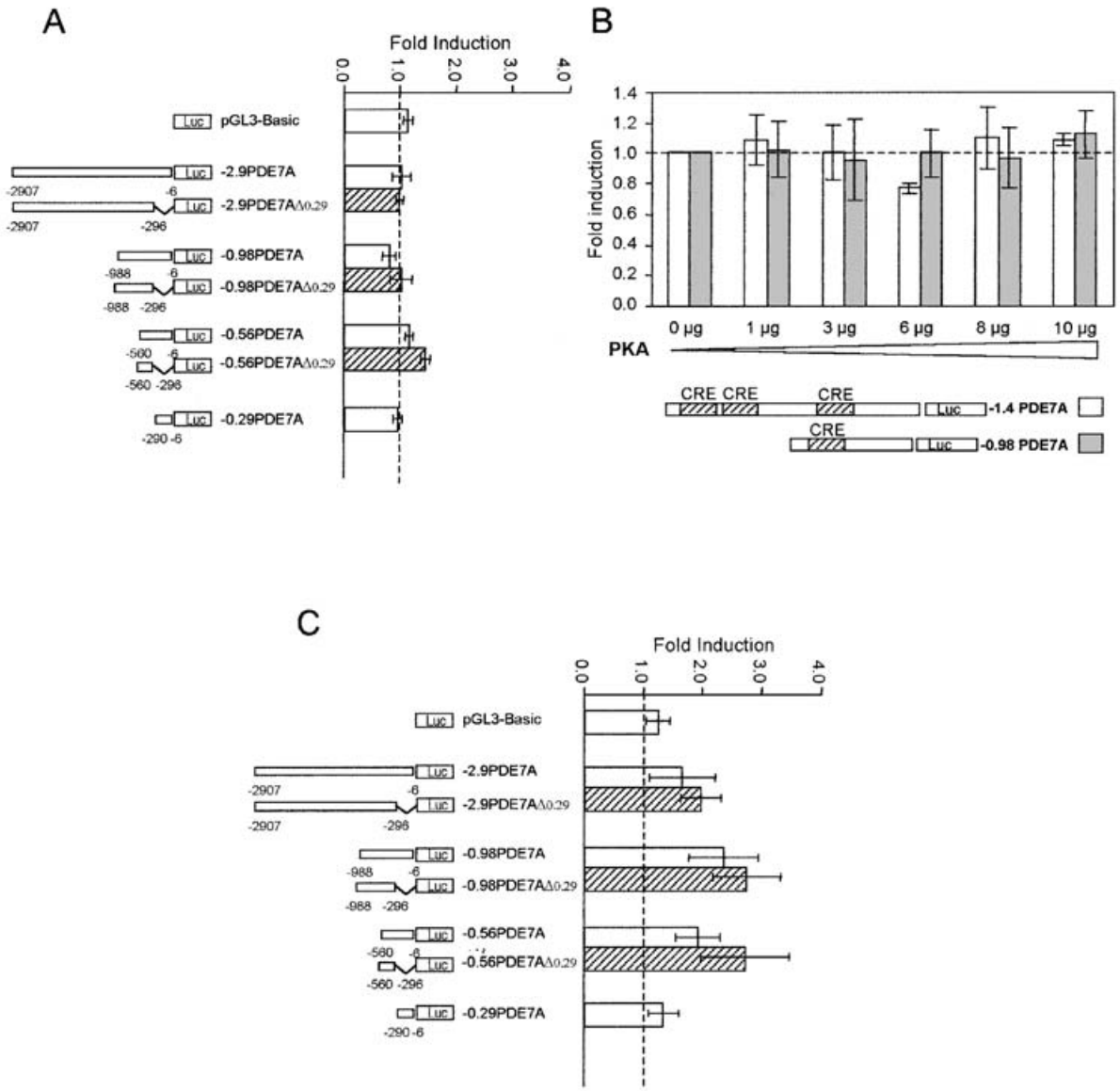

Figure 7 Induction of the h*PDE7A1* promoter by treatment with db-cAMP and PMA

The fold induction obtained after treatment for 24 h with 2 mM db-cAMP (**A**) or 20 nM PMA (**C**) is presented for the promoter constructs indicated on the left. (**B**) The effect of over-expressing the catalytic subunit of PKA on the activity of the indicated promoter constructs. Luciferase activities were determined upon the corresponding treatment and normalized with respect to that of the same construct obtained in the absence of any treatment. Results correspond to means ± S.D. from four (**A**) and at least six (**B** and **C**) independent experiments.

h*PDE7A1* and h*PDE7A3* might share a common promoter

hPDE7A isoforms are differentially expressed in some tissues. hPDE7A1/hPDE7A3 are expressed in lymphoid cells [8,9,11,12] and, although with different kinetics, they are both induced upon T-cell activation [9]. On the other hand, hPDE7A2 is predominant in skeletal muscle and heart [8,10,11]. The promoter region described here is also likely to drive transcription of the hPDE7A3 isoform, since a single 5′ RACE product was obtained with primers of the first exon that is common to both hPDE7A1 and hPDE7A3. Others [9] have reported similar results. The apparent differences in inducibility and tissue expression observed [9] could arise from differential splicing regulation or differences in stability of the transcripts. On the other hand, the transcription start site of hPDE7A2 isoform was mapped at the second exon of the h*PDE7A* gene [10], approx. 50 kb away from the +1 position of h*PDE7A1*. Therefore, it is feasible that h*PDE7A2* expression would be driven by a second promoter located at the first intron. Actually, the contribution of differential promoter usage to the generation of isoforms has been demonstrated for h*PDE5A* and h*PDE4D* [3,6]. In these cases, an intronic promoter is responsible for the generation of some of the corresponding variants.

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