Molecular cloning of a novel splice variant of human type IV_A (PDE-IV_A) cyclic AMP phosphodiesterase and localization of the gene to the p13.1-q12 region of human chromosome 19

Yvonne M. HORTON,* Michael SULLIVAN† and Miles D. HOUSLAY‡

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, IBL, University of Glasgow, Glasgow G12 8QQ, U.K.

We have isolated from a human T-cell Jurkat cDNA library a novel human cDNA (2EL) that is closely related to the human type-IV PDE splice variant family 'A' (PDE-IV_A) cDNA characterized previously by us [Sullivan, Egerton, Shakur, Marquardsen and Houslay (1994) *Cell. Signalling* 6, 793–812]; (h6.1, PDE-IV_{A/h6.1}; HSPDE4A7). (PDE stands for cyclic nucleotide phosphodiesterase). The novel cDNA 2EL (PDE-IV_{A/2EL}; HSPDE4A8) contains two regions of unique sequence not found in PDE-IV_{A/h6.1}. These are a distinct 5'-end and a 34 bp insert which occurs within a domain thought to encode the type-IV PDE catalytic site and which can be expected to result in premature truncation of any expressed protein. HSPDE4A8 appeared to be catalytically inactive. Isolation and characterization of a human genomic cosmid clone revealed that 2EL and

h6.1 represent alternative splice variants of the human PDE-IV_A gene. Using a unique sequence found at the 5'-end of the 2EL cDNA, a probe was generated which was used to screen the DNA of human–hamster hybrids. This located the human gene for PDE-IV_A to human chromosome 19. Through both the analysis of genomic DNAs from a human–hamster somatic cell hybrid panel and also using fluorescent *in situ* hybridization, it was shown that the human PDE-IV_A gene is located on human chromosome 19, between p13.1 and q12. This region on chromosome 19 has been shown to be related to genetic diseases such as the autosomal dominant cerebrovascular disease CADASIL, susceptibility to late-onset Alzheimer's disease and changes seen in benign pituitary and thyroid adenomas.

INTRODUCTION

Cyclic AMP (cAMP) and cGMP are both key regulators of signal transduction mechanisms in a wide variety of biological responses. Cyclic nucleotide phosphodiesterases (PDEs) catalyse the hydrolysis of these 3',5' cyclic nucleotides to the corresponding nucleoside 5'-monophosphates. Biochemical and immunological analyses have revealed that the PDEs exist as a complex array of isoforms [1–4]. The recent application of molecular biology techniques has revealed further complexity arising from the existence of multiple genes within single isoform classes and alternative splicing [5,6–8]. At present, there are considered to be seven classes of PDEs [8]. These are, the type-I, Ca²⁺/calmodulin (CaM)-dependent PDEs; the type-II, cGMP-stimulated PDEs; the type-III, cGMP-inhibited PDEs; the type-IV, cAMP-specific PDEs; the type-V, cGMP-specific PDEs; the type-VI, photoreceptor PDEs and the type-VII cAMP-specific, rolipram-insensitive PDEs. Three different cDNAs have been isolated for the type I, Ca²⁺/CaM-dependent PDEs. These are clearly derived from two different genes, with one expressing two splice variants, and have been confirmed by independent biochemical analyses of this family [9–13]. Only one cDNA clone representing a type II cGMP-stimulated PDE has been isolated, although RNAase protection studies revealed an alternative splice variant with an altered N-terminal domain [14]. This

would be consistent with analyses done on the cytosolic and integral membrane-bound type-II PDEs in hepatocytes, which appeared to differ by virtue of an additional peptide seen in the membrane-bound species upon analysis of ¹²⁵I iodinated tryptic peptides [15]. The type III cGMP-inhibited PDEs appear to be represented by at least two genes [16–18], with both membrane-bound and soluble species identified. Of all the PDE families, however, the most intensely studied have been the type IV cAMP-specific isoforms. This has allowed the identification of four distinct type IV PDE genes, with multiple splice variants having been cloned as cDNAs [5,6,19–26]. The rod and cone photoreceptor PDEs of the type-VI family are represented by three distinct genes: one cone-specific gene and two rod-specific genes, with one of the latter producing two splice variants [27–29]. The type VII class has, to date, only one member, identified by molecular cloning [30] and biochemical studies [31].

The type IV cAMP-specific PDEs are inhibited by the compounds rolipram and Ro 20-1724, a feature which has been used to define this isoform family [1–3,5,6]. Studies of cDNAs isolated from rat and human sources have indicated the presence of four closely related species: A, B, C, and D [5,7,8,26]. The sequences of the proteins encoded by these cDNAs show extremely high homology over the putative catalytic region but little or no homology at their extreme N- and C-termini [5,7,8,26,32,33]. It has been suggested, and demonstrated for the type-IVA species

Abbreviations used: cAMP, cyclic AMP; CaM, calmodulin; PDE, cyclic nucleotide phosphodiesterase; CHO, Chinese hamster ovary; ORF, open reading frame; FISH, fluorescence *in situ* hybridization; hPDE-IV, human type-IV, cAMP-specific phosphodiesterase; hPDE-IV_A, human type-IV PDE splice variant family 'A'; h6.1, a human type-IV PDE splice variant referred to as hPDE-IV_{A/h6.1} (HSPDE4A7); 2EL, a human type-IV PDE splice variant also referred to as hPDE-IV_{A/2EL} (HSPDE4A8).

The nucleotide sequences of hPDE-IV_{A/h6.1} (HSPDE4A7) and hPDE-IV_{A/2EL} (HSPDE4A8) appear in GenBank under accession numbers U18087 and U18088 respectively.

* Present address: Department of Medical Genetics, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, U.K.

† Present address: Fisons Pharmaceuticals, Bakewell Road, Loughborough, Leicestershire LE11 0RH, U.K.

‡ To whom correspondence should be addressed.

Table 1 Nucleotide sequence of 2EL and the predicted translation product

The nucleotides shown in bold italics represent the two regions of unique sequence that are found in 2EL, compared with h6.1. The long ORF starts at the initiation codon at base 333. These sequences have been deposited in GenBank under accession numbers U18088 and U18087 respectively.

1	GG	AGA	GAC	ATG	GAA	AAT	AAG	CCC	TTC	TTC	CTT	TCT	CCC	CTG	GGG	CTC	47
48	GTG	AAC	AGC	TTA	GCT	AAG	AGG	CTG	AGC	TGT	GCT	GGG	GGC	CAG	TCG	TGC	95
96	TGG	TTC	CTA	TCC	TAG	CTT	TGC	CAC	TTA	TGA	GCT	GCA	TGA	TCT	TGG	AGA	143
144	AGT	CAC	A TC	GCC	TCT	CTG	AGC	CTT	GGT	GCC	TTC	ACC	TGC	CAA	ACG	AGA	191
192	TCA	AGT	GCT	TAG	AGC	AAT	A CT	TGG	CAG	ATC	ATA	AGT	ACT	CAA	AAG	ACA	239
240	GTG	GCT	GTG	ATT	GCT	TTC	GCT	ATT	TTG	CAG	CCC	AGT	AGG	ACG	AGG	GCA	287
1																Met	1
288	GTA	CAT	TCT	GTG	CTG	TTG	AAC	CTG	TCA	CCC	ACT	GGG	TGA	GCA	A TA	ATG	335
2	Val	Leu	Pro	Ser	Asp	Gln	Gly	Phe	Lys	Leu	Leu	Gly	Asn	Val	Leu	Gln	17
336	GTG	CTT	CCT	TCA	GAC	CAA	GGC	TTC	A AA	CTC	CTA	GGA	AAT	GTT	CTC	CAG	383
18	Gly	Pro	Glu	Pro	Tyr	Arg	Leu	Leu	Thr	Ser	Gly	Leu	Arg	Leu	His	Gln	33
384	GGC	CCT	GAG	CCT	TAC	AGG	CTT	CTG	ACC	TCT	GGC	CTC	CGT	CTC	CAC	CAG	431
34	Glu	Leu	Glu	Asn	Leu	Asn	Lys	Trp	Gly	Leu	Asn	Ile	Phe	Cys	Val	Ser	49
432	GAA	CTG	GAG	AAC	CTG	AAC	A AG	TGG	GGC	CTG	AAC	ATC	TTT	TGC	GTG	TCG	479
50	Asp	Tyr	Ala	Gly	Gly	Arg	Ser	Leu	Thr	Cys	Ile	Met	Tyr	Met	Ile	Phe	65
480	GAT	TAC	GCT	GGA	GGC	CGC	TCA	CTC	ACC	TGC	ATC	ATG	TAC	ATG	ATA	TTC	527
66	Gln	Glu	Arg	Asp	Leu	Leu	Lys	Lys	Phe	Arg	Ile	Pro	Val	Asp	Thr	Met	81
528	CAG	GAG	CGG	GAC	CTG	TTG	AAG	AA A	TTC	CGC	ATC	CCT	GTG	GAC	A CG	ATG	575
82	Val	Thr	Tyr	Met	Leu	Thr	Leu	Glu	Asp	His	Tyr	His	Ala	Asp	Val	Ala	97
576	GTG	ACA	TAC	ATG	CTG	ACG	CTG	GAG	GAT	CAC	TAC	CAC	GCT	GAC	GTG	GCC	623
98	Tyr	His	Asn	Ser	Leu	His	Ala	Ala	Asp	Val	Leu	Gln	Ser	Thr	His	Val	113
624	TAC	CAT	A AC	AGC	CTG	CAC	GCA	GCT	GAC	GTG	CTG	CAG	TCC	ACC	CAC	GTA	671
114	Leu	Leu	Ala	Thr	Pro	Ala	Leu	Asp	Ala	Val	Phe	Thr	Asp	Leu	Glu	Ile	129
672	CTG	CTG	GCC	ACG	CCT	GCA	CTA	GAT	GCA	GTG	TTC	ACG	GAC	CTG	GAG	ATT	719
130	Leu	Ala	Ala	Leu	Phe	Ala	Ala	Ala	Ile	His	Asp	Val	Asp	His	Pro	Gly	145
720	CTC	GCC	GCC	CTC	TTC	GCG	GCT	GCC	ATC	CAC	GAT	GTG	GAT	CAC	CCT	GGG	767
146	Val	Ser	Asn	Gln	Phe	Leu	Ile	Asn	Thr	Asn	Ser	Glu	Leu	Ala	Leu	Met	161
768	GTC	TCC	AAC	CAG	TTC	CTC	ATC	AA C	ACC	AAT	TCG	GAG	CTG	GCG	CTC	ATG	815
162	Tyr	Asn	Asp	Glu	Ser	Val	Leu	Glu	Asn	His	His	Leu	Ala	Val	Gly	Phe	177
816	TAC	AA C	GAT	GAG	TCG	GTG	CTC	GAG	A AT	CAC	CAC	CTG	GCC	GTG	GGC	TTC	863
178	Lys	Leu	Leu	Gln	Glu	Asp	Asn	Cys	Asp	Ile	Phe	Gln	Asn	Leu	Ser	Lys	193
864	AAG	CTG	CTG	CAG	GAG	GA C	AAC	TGC	GAC	ATC	TTC	CAG	A AC	CTC	AGC	AA G	911
194	Arg	Gln	Arg	Gln	Ser	Leu	Arg	Lys	Met	Val	Ile	Asp	Met	Val	Leu	Ala	209
912	CGC	CAG	CGG	CAG	AGC	CTA	CGC	AAG	ATG	GTC	ATC	GAC	ATG	GTG	CTG	GCC	959
210	Thr	Asp	Met	Ser	Lys	His	Met	Thr	Leu	Leu	Ala	Asp	Leu	Lys	Thr	Met	225
960	A CG	GAC	ATG	TCC	AAG	CAC	ATG	ACC	CTC	CTG	GCT	GAC	CTG	AA G	ACC	ATG	1007
226	Val	Glu	Thr	Lys	Lys	Val	Thr	Ser	Ser	Gly	Val	Leu	Leu	Leu	Asp	Asn	241
1008	GTG	GAG	ACC	AAG	AAA	GTG	ACC	AGC	TCA	GGG	GTC	CTC	CTG	CTA	GAT	AAC	1005
242	Tyr	Ser	Asp	Arg	Ile	Gln	Val	Leu	Arg	Asn	Met	Val	His	Cys	Ala	Asp	257
1056	TAC	TCC	GAC	CGC	ATC	CAG	GTC	CTC	CGG	AAC	ATG	GTG	CAC	TGT	GCC	GAC	1103
258	Leu	Ser	Asn	Pro	Thr	Lys	Pro	Leu	Glu	Leu	Tyr	Arg	Gln	Trp	Thr	Asp	273
1104	CTC	AGC	A AC	CCC	ACC	AA G	CCG	CTG	GAG	CTG	TAC	CGC	CAG	TGG	ACA	GA C	1151
274	Arg	Ile	Met	Ala	Glu	Phe	Phe	Gln	Gln	Gly	Asp	Arg	Glu	Arg	Glu	Arg	289
1152	CGC	ATC	ATG	GCC	GAG	TTC	TTC	CAG	CAG	GGT	GAC	CGA	GAG	CGC	GAG	CGT	1199
290	Gly	Met	Glu	Ile	Ser	Pro	Met	Cys	Asp	Lys	His	Thr	Ala	Ser	Val	Glu	305
1200	GGC	ATG	GAA	ATC	AGC	CCC	ATG	TGT	GAC	AAG	CAC	ACT	GCC	TCC	GTG	GAG	1247

Table 1 (continued)

306	Lys	Ser	Gln	Val	Gln	Ala	Arg	Gly	Ile	Asp	Gly	Arg	Ala	Gln	Gly	Gly	321
1248	AAG	TCT	CAG	GTA	CAG	GCT	CGG	GGC	ATT	GAT	GGA	CGG	GCA	CAG	GGT	GGG	1295
322	Phe	Tyr	***														
1296	TTT	TAT	TGA	CTA	CAT	TGT	GCA	CCC	ATT	GTG	GGA	GAC	CTG	GGC	GGA	CCT	1343
1344	TGT	CCA	CCC	AGA	TGC	CCA	GGA	GAT	CTT	GGA	CAC	TTT	GGA	GGA	CAA	CCG	1391
1392	GGA	CTG	GTA	CTA	CAG	CGC	CAT	CCG	GCA	GAG	CCC	ATC	TCC	GCC	ACC	CGA	1439
1440	GGA	GGA	GTC	AAG	GGG	GCC	AGG	CCA	CCC	ACC	CCT	GCC	TGA	CAA	GTT	CCA	1487
1488	GTT	TGA	GCT	GAC	GCT	GGA	GGA	GGA	AGA	GGA	GGA	AGA	AAT	ATC	AAT	GGC	1535
1536	CCA	GAT	ACC	GTG	CAC	AGC	CCA	AGA	GGC	ATT	GAC	TGC	GCA	GGG	ATT	GTC	1583
1584	AGG	AGT	CGA	GGA	AGC	TCT	GGA	TGC	AAC	CAT	AGC	CTG	GGA	GGC	ATC	CCC	1631
1632	GGC	CCA	GGA	GTC	GTT	GGA	AGT	TAT	GGC	ACA	GGA	AGC	ATC	CCT	GGA	GGC	1679
1680	CGA	GCT	GGA	GGC	AGT	GTA	TTT	GAC	ACA	GCA	GGC	ACA	GTC	CAC	AGG	CAG	1727
1728	TGC	ACC	TGT	GGC	TCC	GGA	TGA	GTT	CTC	GTC	CCG	GGA	GGA	ATT	C		1770

from rat, that such unique domains may be involved in conferring distinct regulatory properties as well as the targeted association of isoforms with membranes [34–36]. The recent isolation and characterization of genomic clones for the rat PDE IV-B and IV-C genes revealed extensive alternative splicing and the presence of two differentially active promoters in the IV-B gene, adding further to the complexity of the mechanisms used to regulate and produce PDE diversity [37]. We have recently isolated [22] a type IV-A cDNA, which we have called h6.1, from a human T-cell library. Expression of this PDE in both COS cells and yeast shows that our isolated cDNA encodes a type IV-A cAMP-specific PDE that is insensitive to Ca²⁺/CaM and cGMP and is inhibited in a simple, competitive fashion by low concentrations of rolipram [38]. The sequence of this cDNA was related to a human PDE IV-A isolated by Livi et al. [24], although base changes would lead to five amino acid differences (see [22,37]). These changes occurred close to the catalytic domain and appeared to give rise to different kinetics of inhibition by rolipram (see [37]). We have suggested that the sequence found in h6.1 reflects that of the native enzyme [22] and, indeed, studies by Bolger et al. [7,26] have demonstrated the isolation of a cDNA which includes a sequence identical with that of h6.1 over the region where the sequence of the cDNA isolated by Livi et al. [24] differs.

In this study we have screened a resting human T-cell library and isolated a novel type IV-A splice variant. This differs from h6.1 by virtue of two regions of unique sequence not found in h6.1 or any other reported PDE cDNA. We found that the protein encoded by the long open reading frame (ORF) of this novel cDNA is inactive as a PDE when expressed in *Saccharomyces cerevisiae* and COS cells. Isolation of a cosmid clone shows that h6.1 and 2EL represent splice variants of one gene. We have used the unique sequence found at the 5'-end of 2EL to show that there is a single human PDE IV-A gene and that it is localized on human chromosome 19.

MATERIALS AND METHODS

cDNA library screening

The human T-cell Jurkat cDNA library made in λ gt11 was purchased from Clontech (Cat. HL1016b). Plaques (5×10^6) were plated and transferred to Hybond-N filters (Amersham International) and hybridized at 65 °C in a solution containing

the human PDE type IV-specific probe described previously [22], $5 \times$ Denhardt's solution (0.1 % polyvinylpyrrolidone/0.1 % ficoll/0.1 % BSA), $6 \times$ SSC (0.9 M NaCl/0.09 M sodium citrate) and sonicated heat-denatured salmon sperm DNA (100 μ g/ml). For library screening, the DNA probe was radiolabelled with [α -³²P]dATP using the T7 Quick Prime Kit (Pharmacia). Filters were washed in $3 \times$ SSC/0.1 % SDS. Positively hybridizing phage plaques were purified and the λ DNA was isolated as previously described [39].

Plasmids, DNA manipulations and sequencing

Purified DNA from positive plaques was digested with *Eco*RI and the insert DNA was subcloned into *Eco*RI digested, dephosphorylated, pUC19, to create plasmid p19-2EL. The insert in p19-2EL was sequenced completely on both strands.

PCR [40] was performed on plasmid DNA (1 ng) in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 200 μ M each of the four deoxyribonucleotides, oligonucleotide primers (0.2 μ M final concentration for both) and 2 units of Taq polymerase (Cetus). Following twenty cycles (30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C) and then incubation at 72 °C for 5 min, the reaction products were analysed by agarose gel electrophoresis and DNA fragments were purified [41].

Plasmid DNA was sequenced by the dideoxynucleotide chain termination method [42–44] using T7 sequencing kits, purchased from Pharmacia, and [α -³⁵S]dATP (Amersham International). *Escherichia coli* K803 was used for plasmid transformation and propagation as previously described [45–46]. *E. coli* Y1090 was used for library screening and λ DNA preparation.

Oligonucleotides and generation of a 2EL-specific probe

The unique 431bp found at the 5'-end of 2EL was amplified in a PCR reaction using the primers 2EL5'-A and 2EL5'-B. The reaction product was sub-cloned into pUC19 (*Eco*RI/*Hind*III) to create the plasmid p19-2EL-5'specific. This plasmid was used to generate the probe used in the subsequent Southern blots. Thus, the oligonucleotide primers used to amplify and subclone the unique sequence from the 5'-end of clone 2EL were: 2EL5'-A, 5'-AGCTAAGCTTGGAGAGACATGGAAAAT-3', and

Table 2 Identity of the unique sequence found in the 5'-end of 2EL

The unique sequence from the 5'-end of 2EL is shown in bold italics. The sequence which is underlined is shared by both 2EL and h6.1. The symbol † denotes the first base of the exon found in h6.1 and ¶ marks the last base of both the 2EL and h6.1 exon.

	10	20	30	40	50	60
5'	GCTACTATGT	GCTGGCCCTT	TCTGAATTTT	GGAGAGACAT	GGAAAATAAG	CCCTTCTTCC
	70	80	90	100	110	120
	TTTCTCCCT	GGGGCTCGT	AACAGCTTAG	CTAAGAGGCT	GAGCTGTGCT	GGGGGCCAGT
	130	140	150	160	170	180
	CGTGCTGGGT	TCCTATCCTA	GCTTTGCCAC	TTATGAGCTG	CATGATCTTG	GAGAAAGTCAC
	190	200	210	220	230	240
	ATCGCCTCTC	TGAGCCTTGG	TGCCCTTCACC	TGCCAAACGA	GATCAAGTGC	TTAGAGCAAT
	250	260	270	280	290	300
	ACTTGGCAGA	TCATAAGTAC	TCAAAAGACA	GTGGCTGTGA	TTGCTTTTCGC	TATTTTGACG
	310	320	330	340	350	360
	CCCAGTAGGA	CGAGGGCAGT	ACATTCTGTG	CTGTTGAACC	TGTCACCCAC	TGGGTGAGCA
	370	380	390	400	410	420
	ATAATGGTGC	TTCTTCAGA	CCAAGGCTTC	AAACTCCTAG	GAAATGTTCT	CCAGGGCCCT
	430	440	450	460	470	480
	GAGCCTTACA	GGCTTCTGAC	CTCTGGCCTC	CGTCTCCACC	AGG†AACTGGA	GAACCTGAAC
	490	500	510	520	530	540
	AAGTGGGGCC	TGAACATCTT	TTGCGTGTGC	GATTACGCTG	GAGGCGGCTC	ACTCACCTGC
	550	560	570	580	590	600
	ATCATGTACA	TGATATTCCA	G¶GTGATGGGG	ACAGCTGGGA	GTGGGCCCGG	GTGAGGGGCT
	CATAGCG	3'-end				

2EL5'-B, 5'-AGCTGAATTCCTGGTGGAGACGGAGG-3'. The *EcoRI* and *HindIII* sites which were used to clone the PCR product using these two primers are underlined.

Oligonucleotide primers used to amplify the unique sequence present at the 5'-end of 2EL in the PCR analysis of the human-hamster somatic cell hybrids were: sense (A) 1794, 5'-GGAGAGACATGGAAAATAAGCCCT-3' and the anti-sense (B) oligonucleotide 1837, 5'-CTGGTGGAGACGGAGGCC-AGAGGT-3'. The sense primer A was complementary to the coding sequence of the gene corresponding to nucleotides 1-24 and the antisense primer B was complementary to the nucleotides 408-431 (Tables 1 and 2). The primers resulted in amplification of a ~ 430 bp fragment.

Southern-blot analysis

The initial assignment to a chromosome was done using a Southern-blot analysis of a panel of 20 Chinese hamster-human hybrids (8 µg/lane) containing various complements of human chromosomes that had been digested with *PstI* (BIOS Laboratories, ScotLab, Livingstone, U.K.). Genomic DNA from human, mouse and hamster (5 µg/lane) were used as controls. Agarose gel electrophoresis was used to separate species before Southern-blot analysis. *PstI* was chosen to digest the DNA from the hybrids as prior analysis, using a test panel, showed that it gave a substantially different hybridization pattern between human and hamster DNAs and produced only one detectable hybridizing band using our specific probe (results not shown). The blots were prehybridized for 30 min at 65 °C in 10% dextran sulphate, 6 × SSC, 1% SDS, 5 × Denhardt's solution and denatured salmon sperm DNA (100 µg/ml). The probe used for Southern-blot analysis was a 431 bp *EcoRI-HindIII* fragment originating from the 5'-region of the 2EL cDNA contained within the plasmid p19-2EL-5'specific. This fragment represents the unique sequence from the 5'-end of 2EL and was isolated from low melting-point agarose (Sigma, U.K.). The method of Feinberg and Vogelstein [47] was used to label the probe with [³²P]dATP (Amersham International) to a specific activity of

1 × 10⁹ c.p.m./µg. The radiolabelled probe was denatured by boiling and added to the prehybridization mix at a final concentration of (1-5) × 10⁶ c.p.m./ml. Hybridization was performed overnight at 65 °C. After hybridization, the blots were washed in 0.5 × SSC/0.5% SDS for 10 min at room temperature, then once in 0.5 × SSC/0.5% SDS for 10 min at 65 °C. The washed filter was then exposed to X-ray film with intensifying screens at -80 °C overnight.

Somatic cell hybrids and cell lines

Hybrid cell lines informative for the long arm of chromosome 19 were used to refine the mapping position. The cell line 5HL9-4 contained only human chromosome 19 [48]. The hybrid cell line, designated 908K1, contains a single derivative (X;19) (q24;q13.2) chromosome with a rearranged 19p arm [49] and hybrid GM89A99c-7 contained the distal region q13.3-qter [50]. These hybrids were derived from a patient with a reciprocal translocation 46,X t(X;19) (q22;q13) karyotype (NIGMS Human Genetic Mutant Cell Repository GM0089, Camden, NJ, U.S.A.). The cell line 2F5 was an irradiation-reduced hybrid containing approximately 2 Mb of DNA derived exclusively from a small region of 19q13.3 [51]. Somatic cell hybrids derived from Chinese hamster ovary (CHO) DNA repair-deficient mutants were 135HL30, 1SHL9 and 9HL5 [52-54]. The cultured hybrid cell lines were immobilized in agarose plugs for use as PCR template DNA. The plugs were stored in 0.5 M EDTA.

PCR amplification from agarose plugs

The storage buffer (0.5 M EDTA) was removed and either one or two plugs for each cell line were placed in a 1.5 ml microcentrifuge tube. The plugs were rinsed in four changes of 10 mM Tris-HCl (pH 8.0) for 15 min without agitation. The plugs were drained and melted briefly at 65 °C. Distilled water was added to a final volume of 500 µl. Molten plug (10 µl) was used for each PCR reaction. Amplification was performed in 50 mM KCl/10 mM Tris-HCl/1.5 mM MgCl₂ containing 200 µM of each dNTP,

25 pmol of each primer and 5 units of Taq DNA polymerase (Promega) in a final volume of 50 μ l. The samples were overlaid with 100 μ l of mineral oil to prevent condensation. The amplification protocol included an initial denaturation at 94 °C for 5 min, then 30 cycles (1 min at 94 °C, 2 min at 55 °C, 3 min at 72 °C) and a final extension period of 15 min at 72 °C. The reaction products were analysed by electrophoresis through a 2% agarose gel. Assignment of a target sequence to a specific region of the chromosome was accomplished by comparing human-specific amplified products in an ethidium bromide stained gel with DNA products being visualized under UV light.

Fluorescence chromosomal *in situ* hybridization (FISH)

Human metaphase cells were prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes. The ORF of the human PDE type IV-A h6.1 cDNA was isolated from the plasmid pSVsport-6.1 [22] and biotinylated (1 μ g) with biotin-11-dUTP (Gibco-BRL) by nick translation, adapted from the previously described method [55]. The probe was hybridized to chromosome preparations overnight at 37 °C. The signal was detected with fluorescein isothiocyanate-conjugated avidin and amplified according to the method described by Carter [56]. The chromosomes were counterstained with propidium iodide and analysed using a Zeiss Axiophot microscope with a cooled charge-coupled device camera.

RESULTS AND DISCUSSION

Isolation of a novel human PDE cDNA from a Jurkat T-cell cDNA library

A cDNA library made from resting human Jurkat T-cells was screened with a PDE-specific probe generated by PCR as described previously [22]. From the library screen one plaque gave a positive hybridization signal after repeated rounds of plaque purification. The DNA from the positive plaque (2EL), was purified and digested with *Eco*RI and the insert was cloned into *Eco*RI-digested pUC19 to generate p19-2EL. The insert of p19-2EL was sequenced completely in both orientations (Table 1). The cDNA was 1770 bp in length and appeared to be a partial clone, having been digested internally with *Eco*RI during library construction as a result of incomplete methylation of *Eco*RI sites in the cDNA population. The sequence of 2EL revealed close homology overall with the previously isolated human PDE-IV_A cDNA h6.1 [22]. Indeed, sequence alignment with h6.1 revealed that 2EL contains two regions of unique sequence with the rest

of the sequence of 2EL being identical with that of h6.1. The regions of unique sequence found in clone 2EL consisted of 431bp located at the extreme 5'-end and an internally located 34 bp insert (Table 2). The largest ORF in 2EL encodes a protein of 323 amino acids and starts at an initiation codon located within the unique sequence at its 5'-end (Table 1). The initiation codon of the long ORF is the fourth ATG from the 5'-terminus of 2EL (Table 1).

Comparison of the putative proteins encoded by 2EL and 6.1 shows that 2EL has 33 novel amino acids at the N-terminus (Table 2). After this point, the two clones share the same 275 amino acids where the premature termination of the 2EL ORF would be expected to occur as a result of a frameshift caused by the 34 bp insert. Thus 2EL has a unique sequence of 14 amino acids which form its novel C-terminus, whilst the ORF of h6.1 encodes, instead, some 243 amino acids forming this C-terminal end [22]. Expression of the engineered long ORF of h6.1 in the *S. cerevisiae* strain YMS5X, has been shown [22] to rescue this strain from heat shock sensitivity through the expression of novel PDE activity which can be determined in such engineered cells [38]. YMS5X [38] does not express any PDE activity, as the endogenous yeast PDE genes have been disrupted [22]. However, we were unable either to observe PDE activity in, or to rescue from heat shock, YMS5X cells which had been engineered to express the long ORF of 2EL (results not shown). Furthermore, in contrast to studies done with h6.1 [22], we were unable to observe any increase ($< 5\%$ change; $n = 3$ separate experiments with each plasmid) in the PDE activity of COS cells transfected with either of the vectors pSPORT or pSVL containing the long ORF of 2EL (results not shown).

We have previously reported the isolation of a human cDNA clone h6.1 that encodes a type IV_A cAMP PDE [22]. The DNA probe used to isolate the type IV_A PDE cDNA was generated using a human Jurkat T-cell cDNA library as template in a PCR reaction carried out with human PDE type-IV specific primers. When the DNA fragment generated by PCR was cloned and sequenced [22] we found that the sequence was identical in places with a previously published human cAMP PDE cDNA [24], but contained an insert of 34 bp that was identical in position and sequence with that reported here in the 2EL cDNA. At that time we considered the possibility that the 34 bp insert in our PCR-generated probe might have arisen through an artefact of the PCR process. However, the fact that we found this same sequence in our isolated 2EL cDNA clone would appear to eliminate such an explanation. Of course, it is also possible that the unique sequences found in 2EL arose through cloning errors during library construction. We have, however, isolated and sequenced

Table 3 Exon usage by 2EL and h6.1: location of a 34 base insert

The exon used by h6.1 is underlined. The extended exon used by 2EL contains, in addition to the sequence found in h6.1, the extra 34 bases that are shown in bold italics. The symbol † denotes the last base of the h6.1 exon and ¶ marks the last base of the 2EL exon.

5'-end

10	20	30	40	50	60
TTTCCCTGT	GCCCCAACCC	CATGCAGGTC	CTCCGGAACA	TGGTGCACTG	TGCCGACCTC
70	80	90	100	110	120
AGCAACCCCA	CCAAGCCGCT	GGAGCTGTAC	CGCCAGTGGA	CAGACCGCAT	CATGGCCGAG
130	140	150	160	170	180
TTCTTCAGC	AGGGTGACCG	AGAGCGCGAG	CGTGGCATGG	AAATCAGCCC	CATGTGTGAC
190	200	210	220	230	240
AAGCACACTG	CCTCCGTGGA	GAAGTCTCAG [†]	GTACAGGCTC	GGGGCATTTGA	TGGACGGGCA
250	260	270	280	290	
CAGGAGTGAGT	CTCCCCAGCC	CATCTTGGCC	TGAAGTCTGT	AGGCCCAAGGA	GCTCCT

3'-end

Figure 1 Intron/exon boundaries of the PDE type-IVA gene

A schematic representation of the intron/exon boundaries identified in a human genomic cosmid clone over the region where the alternative splicing ensues, leading to the production of the splice variants identified in this study, namely 2EL, and previously [22,38], namely h6.1. The open boxes indicate exons that are shared by both 2EL and h6.1 and the diagonally hatched boxes exons that are unique to 2EL. Note that the 34 bp insert exon occurring at the 3'-end of 2EL can be predicted to cause premature termination and lead to a truncated species with a novel C-terminus. Note that the differences in splicing will also lead to a distinct 5'-end for 2EL and hence a novel N-terminus for this splice variant. The cross-hatched box indicates an exon which is unique to h6.1 and forms part of its unique N-terminal domain. The probes and PCR primers used to probe the PDE type-IVA gene were formed from sequences within the unique 5' exon found in 2EL.

Table 4 Chromosomal assignment of human type IVA PDE

A panel of Chinese hamster-human hybrids containing various complements of human chromosomes that had been digested with the restriction enzyme *Pst*I were analysed by Southern blotting using a 2EL-specific probe (see Materials and methods section; Figure 2). The data show the number of hybrids in which the specified human chromosome and thus the specific bands (Figure 2) were both present (+/+) or both absent (-/-). It also shows the number of hybrids where the specified human chromosome was absent but the specific band was present (+/-) or where the band was absent but the chromosome was present (-/+).

Chromosome	Concordant		Discordant		Discordancy (%)
	+/+	-/-	-/+	+/-	
1	3	13	3	1	20
2	0	13	6	1	35
3	0	12	6	2	40
4	1	13	5	1	30
5	6	6	0	8	40
6	1	13	5	8	30
7	2	14	4	0	20
8	0	11	6	3	45
9	0	13	6	1	35
10	0	13	6	1	35
11	0	13	6	1	35
12	2	14	4	0	20
13	5	14	1	0	5
14	4	12	2	2	20
15	2	14	4	0	20
16	0	12	6	2	40
17	0	12	6	2	40
18	1	12	5	2	35
19	6	14	0	0	0
20	1	13	5	1	30
21	4	13	2	1	15
22	2	13	4	1	25
X	0	12	6	2	40
Y	2	13	4	1	25

part of a human genomic cosmid clone containing the PDE-IV_A gene (M. Sullivan, unpublished work). This revealed that the two regions of unique sequence in 2EL along with 6.1-specific sequences were present in the one cosmid clone. Comparison of the cDNA sequences with the genomic sequence allowed mapping of the exons in the gene and assignment of exon/intron bound-

Figure 2 Probing of human × hamster somatic cell hybrid restricted DNA fragments with a unique 5' probe for human type-IVA PDE

Filter hybridization analysis of type IV PDE. DNAs were digested with *Pst*I and Southern-blot analysis was performed with the probe 2EL-5'-specific. Lanes 2 and 15 contained total human DNA (band present). The hybrids in lanes 8, 10, 11, 19, 23 and 26 contained human chromosome 19 (band present). The hybrids in all other lanes contained chromosomes other than 19. Lanes 1 and 14 contained hamster DNA, lane 13 mouse DNA and lane 12 contained no DNA (blank). A positive reaction was indicated by the identification of a 1.7 kb fragment as indicated.

aries (Table 3; Figure 1), which followed the consensus rules [57]. It is apparent, therefore, that 2EL and the previously reported cDNA, h6.1, represent alternative splice variants of the human PDE-IV_A gene.

Chromosomal localization of the human type IV-A PDE gene

Chromosome localization of the type IV-A PDE gene was accomplished utilizing Southern blots of hamster × human somatic cell hybrid DNAs. Analysis of restricted DNA from 20 somatic cell hybrids revealed 100% concordance (Table 4) between the presence of human chromosome 19 and hybridization of the 430 bp 2EL-5'-specific probe (see Materials and methods section) to a 1.7 kb *Pst*I restriction fragment. A single human-specific band was observed (Figure 2). No discordancies were found for the presence of the gene on chromosome 19 and the degree of concordance for the remaining chromosomes was between 5 and 40% (Table 4). This unambiguously assigns PDE type IV-A to chromosome 19. No hybridizing band was observed for Chinese hamster or mouse parent cell lines (Figure 2).

Localization of the type-IVA PDE gene to chromosome 19 was confirmed by FISH, conducted on metaphase chromosome preparations. In these studies we used the plasmid pSVsport-6.1, which contains the long ORF of hPDE-IV_{A-h6.1} (h6.1; HSPDE4A7), as probe [22]. This plasmid has been shown previously by us [22] to lead to the expression of a novel type-IV PDE activity in transient transfections done in COS cells. Clear signals were present on each of the chromosome 19 homologues in the centromeric region with a low fluorescent background signal (results not shown). However, it was difficult to refine the

Figure 3 Schematic representation of the regions of chromosome 19 in the somatic cell hybrids

A schematic representation of chromosome 19 which delineates the regions of this chromosome which are found within the particular somatic cell hybrids probed by PCR analysis using primers specific for the unique 5'-end of 2EL (see Figure 1). The lines with arrows indicate the extent of the chromosome incorporated in each of the hybrids. Those represented by the broken lines are hybrids which showed as positive for the analysis and those with a solid line as negative (see Figure 4). Thus, (a) 908K1 contained a single derivative (X;19)(q24;q13.2) chromosome with a rearranged 19p arm [49]; (b) 9HL5 was derived from CHO DNA repair-deficient mutant [54]; (c) 5HL9-4 contained only human chromosome 19 [48]; (d) 1SHL9 was derived from CHO DNA repair-deficient mutant [53]; (e) 2F5 was an irradiation-reduced hybrid containing approximately 2 Mb of DNA derived exclusively from a small region of 19q13.3 [51]; (f) 135HL30 was derived from CHO DNA repair-deficient mutant [52] and (g) GM89A99c-7 contained the distal region q13.3-qter [50].

localization, due to the similarity in size of the long and short arms of chromosome 19. Thus we utilised PCR amplification of somatic cell hybrid DNAs that contained different regions of chromosome 19 [58], enabling intervals across 19q to be defined (Figure 3). This was done using 2EL-specific primers made to the 5' and 3' regions of the 431 bp 2EL-5'-specific probe (see Materials and methods section). DNA from hamster (CHO-LA) cell lines was not amplified (Figure 4), confirming the absence of extensive cross-species homology to this type-IV PDE subfamily. A specific PCR product, of approximately 430 bp (Figure 4), was amplified from total human genomic DNA. A faint fragment at around 740 bp was also observed in these experiments (Figure 4, lane 2) and, in some instances, a faint band at ~227 bp. The 430 bp fragment was observed (Figure 4, lane 6) in the chromosome 19-containing hybrid 5HL9-4 [48], providing independent confirmation of the localization of the PDE type-IVA gene to chromosome 19. Hybrids 908K1, 9HL5, 135HL30 and 1SHL9 all contain overlapping regions of chromosome 19 (see Figure 3). However, a 430 bp PCR product was only obtained from amplification of the hybrid 1SHL9 (Figure 3, lane 7). The hybrid 1SHL9 contains (Figure 3; hybrid d) the most proximal region of the long arm of chromosome 19 [53], from q12 to p13.2, which indicates that the 2EL-specific sequence from the type-IVA PDE gene is localized between q12 and p13.2 on human chromosome 19. None of the hybrids were informative for the short arm.

Figure 4 PCR amplification of somatic cell hybrid DNAs that contained different regions of chromosome 19 using 2EL-specific primers

This was done using 2EL-specific primers as described in the Materials and methods section using the various hybrid cell lines described in Figure 3. The oligonucleotide primers used to amplify the unique sequence were based upon the unique 5'-end of 2EL. The sense primer was complementary to the coding sequence of the gene corresponding to nucleotides 1–24 and the antisense primer B was complementary to the nucleotides 408–431 (Table 2). Lane 1, DNA from CHO cells; lane 2, human DNA from HeLa cells; lane 3, hybrid 908K1; lane 4, hybrid 9HL5; lane 5, hybrid 2F5; lane 6, hybrid 5HL9-4; lane 7, hybrid 1SHL9; lane 8, hybrid GM89A99c-7; lane 9, hybrid 135HL30. The location of the amplified species at ~430 bp is shown.

CONCLUSION

We have described the isolation and characterization of a cDNA (2EL) representing a novel human PDE-IV_A splice variant. With the exception of two regions of unique sequence, the sequence of 2EL is identical with a human IV_A cDNA previously described and called h6.1 [22]. 2EL contains a unique sequence at the 5'-end and an insertion of 34 bp towards the 3'-end. The presence of the 34 bp insert within the ORF can be expected to result in a frameshift and, hence, the premature termination of the ORF. This disruption occurs within a domain which is highly conserved in all type-IV PDEs and which is believed to form the catalytic core of the enzyme [32,33]. 2EL would thus be expected to produce a protein which does not exhibit PDE activity. This would appear to be the case from the observations that we have made here in this study and which are thus consistent with the notion that disruptions in such a domain disrupt catalytic function [32].

2EL is not the first splice variant of the human PDE-IV_A gene to be described which can be expected to lead to an inactive protein product in mammalian cells. For, in this regard, Bolger et al. [26] have characterized a PDE type-IVA splice variant called TM3. This splice variant was originally isolated by virtue of its ability to complement the heat shock sensitive phenotype of a *S. cerevisiae* strain that was rendered devoid of all PDE activity by targeted disruption of the two yeast PDE genes. However, subsequent sequence analysis of TM3 revealed that its message would not be expected to be expressed as the protein product that functions in yeast, without invoking irregular translational initiation in human cells [26]. It is possible then that both TM3 and 2EL represent non-functional splice variants of the human PDE-IV_A gene. Indeed, unless the unique sequences found in 2EL and TM3 represent mutually exclusive exons, splice variants of the human PDE-IV_A gene, in addition to TM3 [26], 2EL (this study), PDE-46 [26] and h6.1 [22], can be expected to be found.

It remains to be seen, however, if either 2EL or TM3 encode proteins which are expressed under physiological conditions. One possibility is that the formation of non-functional splice variants may represent the products of a mechanism used to

control the level of mRNAs for functional PDEs. Thus the production of an mRNA, by alternative splicing, that does not encode a functional protein could represent a mechanism that controls the level of expression of the functional gene product (PDE activity) by lowering the intracellular concentration of mRNAs producing functional PDEs. Indeed, the production of a non-functional splice variant is used to control sexual development of the fruit-fly *Drosophila melanogaster* (see [59] for review). Differential splicing of the *Sex-lethal* gene produces a non-functional mRNA in male flies, whilst female flies produce an mRNA from the same gene that encodes a functional gene product. This 'on-off' switch controls sex determination during development and is determined by alternative splicing that produces functional or non-functional transcripts (see [59] for review). An alternative possibility is that the splice variants which do not exhibit PDE activity may be expressed as proteins which form complexes with the functional species and serve to alter their functioning. This may, for example, take the form of either conferring distinct regulatory properties or, even, targeting such multimers to intracellular membranes. Indeed, it is of interest that whilst the products of the rat type-IVA PDE gene appear to be membrane associated [34,36,60] through unique N-terminal targeting domains, the products of the human type-IVA PDEs, which have been analysed to date by expression in COS cells, appear to form cytosolic species [22,26].

By probing DNAs from a panel of human-hamster somatic cell hybrids with a probe formed from the unique sequence present at the 5'-end of 2EL, we were able to assign the human PDE type-IV_A gene to chromosome 19, as indicated before by us [61] and recently noted by others [62]. Our PCR analysis, reported here, of a somatic cell hybrid which contained only human DNA from chromosome 19, provides independent confirmation of such a chromosomal assignment. We were then able to define further the location of this gene to a region between q12 and p13.2 on chromosome 19, using PCR amplification of somatic cell hybrid DNAs that contained different regions of human chromosome 19. This allowed mapping to the p13.1-q12 region, with FISH studies implying a location at the short arm within this region. A number of genes have been mapped to chromosome 19 [63-65] and, located close to the gene for human PDE type-IV_A (HSPDE4A). These include those for the insulin receptor (p13.3-p13.2), erythropoietin receptor (p13.3-p13.2), thromboxane A2 receptor (TBXA2R) (19p13.3), the *ras*-associated protein RAB 3a (p13.2), ICAM-1 (p13.2-p13.1), the lipoprotein receptor (p13.2) and lysosomal α -D-mannosidase B (p13.2-q12), for example. It is possible that certain genetic diseases could be associated with the HSPDE4A on chromosome 19 [63,64]. Changes in the levels of intracellular cAMP might be expected to have profound effects on cellular functioning and could thus either underlie or contribute to the molecular pathology of certain disease states. It is thought, for example, that type-IV PDEs may play major roles in brain functioning as certain type-IV PDE inhibitors can serve as anti-depressants [66], and distinct regional distributions for the expression of type-IV-A and -B sub-types and their splice variants have been noted in brain [35]; M. D. Houslay, unpublished work]. It is then of interest that a new autosomal dominant cerebrovascular disease (CADASIL), which is associated with ischaemic strokes, has been assigned [67,68] within the interval p13.2-p12. This same gene has also been suggested as playing a role in migraine [69]. Additionally, susceptibility to late-onset Alzheimer's disease also appears to be associated with chromosome 19, with a study mapping this to the centromeric q13.1 region [70]. In many cells, elevation of intracellular cAMP levels appears to inhibit cell growth and proliferation [71], probably by inhibiting *raf* activation [72].

However, in the pituitary and thyroid, elevation of cAMP levels stimulates the proliferative response [71], with activating mutations in the stimulatory guanine nucleotide regulatory protein G_s being oncogenic [73]. A potentiation of the proliferative response might be expected upon any reduction in PDE activity. It is then of interest to note that certain benign pituitary [74] and thyroid [75] adenomas have been shown to exhibit either structural or numerical aberrations in chromosome 19. The chromosomal localization of the human type IV_A PDE gene thus opens new perspectives for the analysis of this gene family. Indeed, additional markers for the same sub-region can now be used to determine the linkage and order of this gene along the chromosome arm. Probes from the 5'- and 3'- ends of the gene, used in combination with long-range restriction analysis, may also help establish both the chromosomal orientation of the gene and the physical distance between neighbouring genes.

The type-IV PDE family, although very similar in terms of inhibitor and substrate binding properties, related in catalytic function due to similarity in sequence over their central domain, shows remarkable differences as regards chromosomal localization [62], distribution [1,7,35,60], membrane association [34-36] and forms expressed due to multiple splicing [5,7]. It is tempting to infer from such observations that the expression of type-IV PDE forms may be tailored so as to confer distinct functional attributes in particular cells. It will thus be of interest to determine the functional role of the various splice variants of the human type-IVA PDE gene and to see if changes in their function or levels of expression provide the underlying molecular pathology of disease states by perturbing the cAMP signalling pathway in specific cells.

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