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Identification and distribution of different mRNA variants produced by differential splicing in the human phosphodiesterase 9A gene

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Abstract

The transcript population of the human gene coding for a cGMP-dependent phosphodiesterase (PDE9A) has a complex structure. There is a high level of mRNA in intestinal and prostate tissues, a low level in blood, and intermediate in other tissues. More than 20 different variants produced by differential splicing have been observed and new exons have been identified both by PCR amplification and by the analysis of available EST sequences. In all cases the transcriptional start site is the same and no differential splicing is found in the exons coding for the catalytic domain of the protein. In some cases the protein produced by splice variants is truncated. The distribution of the splice variants is not homogeneous among the different tissues studied. The human, but not the mouse, PDE9A gene appears to have a complex regulation of expression by different isoforms.

Keywords: cGMP phosphodiesterase; PDE9A; Alternative splicing

Mammalian cyclic nucleotide phosphodiesterases (PDEs) catalyse the hydrolysis of cyclic adenosine 3′,5′-monophosphate (cAMP) and/or cyclic guanosine 3′,5′-monophosphate (cGMP), which are ubiquitous second messengers, responsible for transducing the effect of a variety of extra-cellular signals [1,2]. At least 25 genes coding for cyclic nucleotide PDEs have been identified in mammals. These genes have been classified in 11 families based on several functional and structural characteristics [3], and encode more than 50 different isoforms through alternative splicing as it has been recently reviewed [1]. The physiological implication of the diversity of PDE genes and isoforms produced by each gene is not yet known.

The PDE9A gene encodes a cGMP-specific high-affinity PDE, which seems to be expressed in all tissues examined except blood [4,5]. The human PDE9A gene is located on 21q22.3 and has at least 20 exons spanning 122 kb. Because of its mapping position and its contribution to the regulation of the steady-state cellular level of cyclic nucleotides, PDE9A is a possible candidate for genetic diseases mapped on 21q22.3, such as bipolar affective disorder. Furthermore, its overexpression might be involved in Down's syndrome [4]. The mouse PDE9A gene is located on chromosome 17. It has a high level of expression in kidney and lower levels in liver, lung, and brain [6].

Recent studies have shown that PDE9A mRNA is widely distributed throughout the rat brain with varying regional expression. A high mRNA level was found in the basal forebrain, olfactory bulb, hippocampus, and cerebellum; regions known to be associated with the regulation of behavioural state, olfactory systems, motor control, and learning. An overlap was also observed in the pattern of expression between soluble guanylyl cyclase (sGC), NO synthase, and PDE9A mRNA in the rat brain, indicating a possible role of NO-cGMP signalling pathway in these physiological functions [7].

Four different human PDE9A mRNA transcripts have been so far identified (PDE9A1, PDE9A2, PDE9A3, and PDE9A4), which are produced as a result of alternative splicing of 5′ exons [4]. In higher eukaryotes, alternative splicing is a mechanism to produce biological complexity, allowing a combinatorial explosion in the number of possible proteins with no more than

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40,000 genes per genome. It has been observed that ~40% of human genes undergo alternative splicing [8] and there may be up to about 3.2 mRNA transcripts per gene [9]. Moreover, PDEs encoded by alternatively spliced mRNA were reported to differ in their regulation by kinases [10] and associated proteins [11] and to be accumulated upon treatment by specific factors [12]. Here we report the cloning and characterisation of 16 new additional splice variants of PDE9A, which differ in the exon combination of their 5′ mRNA region. The variants are present in both cell cultures and human tissues as observed by PCR and EST analysis.

Materials and methods

Cell culture. HeLa cells (human cervix carcinoma cell line) were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium with 10% foetal bovine serum. Jurkat cells (human T cell leukemia cell line) were cultured, harvested, and resuspended as previously described [13]. Caco2 cells (human colon adenocarcinoma cell line) were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium with 10% foetal bovine serum, 2 mM L-glutamine, and 1% non-essential amino acids.

RNA blot and dot blot analyses. An oligodeoxyribonucleotide was used as the probe for PDE9A RNA blotting. It is complementary to the sequence between nucleotides 541 and 587, which is present in all PDE9A splice variants identified so far. The oligonucleotide was ^{32}P-radiolabelled and the RNA blot analysis was as previously described [14,15]. Total RNA from HeLa, Jurkat, and Caco2 cells was isolated using the Ultraspect RNA Isolation System (Biotecx Laboratories, Houston). For RNA blot analysis, 15 μg of HeLa and Jurkat total RNA was electrophoresed in a formaldehyde gel and transferred to a Hybond N^+ nylon membrane (Amersham, Piscataway, NJ) overnight using 20× standard saline citrate (SSC) [16].

A PCR-radiolabelled amplified fragment from the fifth PDE9A exon (nucleotides 265–397) was used as the probe for Human Multiple Tissue Expression Array (Clontech, Palo Alto, CA) hybridisation (primers d9A1-r5, Table 1). Hybridisation was performed according to the manufacturer's protocols. The membranes for RNA blot and dot blot were exposed to X-ray film at −70 °C and results were quantified with Phosphoimager software (Quantity One Software, Bio-Rad).

Reverse transcriptase-PCR amplification and 5′ RACE. Retrotranscriptase was performed on total RNA of HeLa, Jurkat, and Caco2 cells to optimise the PCR conditions for specific amplification of alternative splice forms of PDE9A. First, the primer rRT-Nt (Table 1) was used for retrotranscription with M-MLV RT (Promega, Madison, WI), followed by PCR using r9A and d9A primers. Finally, a PCR-nested reaction was performed using r9Anest and specific splice variant primers (primers are shown in Table 1). The amplified products were cloned using the TA vector pGEM-T Easy (Promega, Madison, WI) and sequenced in an ABI Prism 377 sequencer.

Eight pools of samples of cDNA from different human tissues—colon, ovary, peripheral blood leukocyte, prostate, small intestine, spleen, testis, and thymus—available from Clontech (Multiple Tissue cDNA Panels. Clontech, Palo Alto, CA) were used for PCR. This was using the same primers and conditions previously optimised to show the tissue distribution of the different PDE9A splice variants.

To confirm that the 5′ end of the human PDE9A transcript had been obtained, a 5′ RACE reaction was performed on total RNA of Caco2 cells using the GeneRacer Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The primer rRT-Nt was used for retrotranscription and the PCR amplification was then performed using primers r9A and GeneRacer 5′ primer (primers in Table 1). The amplified products were cloned using the TA vector pGEM-T Easy (Promega, Madison, WI) and sequenced.

GenBank Accession Nos. The sequences of the new human PDEA splice variants have been deposited in GenBank with Accession Nos. as follows. PDE9A5: AY196299; PDE9A6: AY196300; PDE9A7: AY196301; PDE9A8: AY196302; PDE9A9: AY196303; PDE9A10: AY196304; PDE9A11: AY196305; PDE9A12: AY196306; PDE9A13: AY196307; PDE9A14: AY196308; PDE9A15: AY196309; PDE9A16: AY196310; PDE9A17: AY196311; PDE9A18: AY196312; PDE9A19: AY196313; and PDE9A20: AY196314.

Results and discussion

Accumulation of PDE9A mRNA: RNA and dot blot analyses

In order to find an appropriate system to study the accumulation of PDE9A mRNA and its population of splice variants, total RNA of HeLa and Jurkat cells was isolated. An RNA blot was then hybridised with a

Table 1
Sequences and positions of oligonucleotide primers used in this study

Name	Position	Sequence
rRT-Nt	816–837	CAG GCA GCT CAG CAT CTC ATT G
d9A	42–63	CAA GGC CAT CTA CCT GGA CAT C
r9A	789–810	CCA AAG CCA GAC GTC AAA GGT C
r9Anest	705–724	GTC GAG GAG TCA ACT TCT TG
d9A1	265–283	CAA CTC TCC GCT GGT GTC G
d9A2.1	Boundary exon 2–3	CAT TCA GAA GCA CTC CGT AC
d9A3.1	Boundary exon 1–4	CAT TCA GAA GCA CTC CGT AC
d9A4.2	Boundary exon 4–7	GCA ACT CTC CGA GCA TTC AA
d9A6	Boundary exon 1–3	CAT TCA GAA GGA ACA CGA CC
d9A8	Boundary exon 1–5	CAT TCA GAA GCT GGT GTC G
d9A9	Boundary exon 2–4	CTG CCT CGC ACT CCG TAC
d9A10	Boundary exon 1–7	CGC ATT CAG AAG AGC ATT C
r5	376–397	CTA CCT GTC CAC TTT CAA ATG C

The positions of the oligonucleotide are according to PDE9A1 sequence (GenBank Accession No. AF067223) or to PDE9A exon structure.

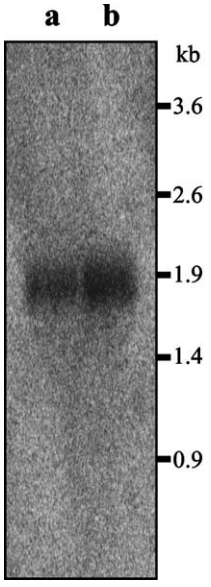

Fig. 1. RNA blot analysis of the human PDE9A transcript. Total HeLa (a) and Jurkat (b) RNA was hybridised with ^{32}P-radiolabelled oligonucleotide probe (nt 541–587). A band of approximately 2 kb is seen.

^{32}P-radiolabelled oligonucleotide (nt 541–587) probe. A band was found around 1.9 kb (Fig. 1), in accordance with the expected size of the PDE9A mRNA. The band often appears broad or as a smear, which may indicate the existence of several mRNAs for the same gene.

The accumulation pattern of the PDE9A1 transcript, the most abundant splice variant described so far, was examined in human tissues by dot blot (Human Multiple Tissue Expression Array, Clontech) analysis (Table 2). A ^{32}P-radiolabelled 132 bp PCR fragment of the PDE9A1 cDNA encoding a large region of the 5th exon (nt 265–397) was used as a probe. As shown in Table 2, the human PDE9A1 transcripts are particularly abundant in prostate, colon, rectum, foetal brain, foetal kidney, and intestine. Moderate expression was observed in other tissues such as cerebellum and forebrain. Nevertheless, some tissues had poor PDE9A mRNA accumulation, such as liver, pancreas, peripheral blood leukocyte, skeletal muscle, and bone marrow. In some cases these are tissues where other PDE genes have high levels of mRNA accumulation, such as PDE7B2 [17] and PDE11A3 [18] in testis, PDE11A4 in prostate [18], and PDE11A1 in skeletal muscle [19]. Other examples of high levels of specific PDE gene expression have been reviewed [1,20,21].

Identification of new splice forms of PDE9A

Alternative splicing has been proposed as an important mechanism for regulating the expression of human PDEs. The existence of multiple splice variants has been described for most human PDE genes [1]. In the case of PDE9A, four variants have already been described in the 20 exon structure depicted in Fig. 2. In order to study the presence of specific variants in different human tissues, RT-PCR was used on total RNA from HeLa, Jurkat, and Caco2 cells with N-terminus PDE9A-specific primers (see Table 1). When this experiment was carried out, at least 16 additional unexpected bands were amplified. The cloning and sequencing of these bands gave previously undescribed cDNAs that may correspond to new splice variants of PDE9A (see Fig. 3). These splice variants revealed a new combination of previously described PDE9A exons and two new exons, all of them involving the 5′ cDNA region. No alternative splicing involving the C-terminal part of the protein, where the catalytic domain is present, was found, as seen by the analysis of available ESTs.

Fig. 3 shows a schematic representation of the previously described four splice variants of PDE9A (PDE9A1–4) and the 16 new splice variants described in this paper (PDE9A5–20). PDE9A5 has a new combination of the previously described exons plus a new exon (3b) between exons 3 and 4, at nt position 46,705–46,781 (Fig. 2) in the genomic sequence (GenBank Accession No. AB017602). This new exon 3b is also present in a human EST from B-cell (chronic lymphatic leukaemia, GenBank Accession No. AI492065). PDE9A19 also has a new exon (4b) between exons 4 and 5, at nt position 73,846–73,925 in the genomic sequence (Fig. 2). This 4b exon, in contrast, has not been found in any of the EST databases searched. The splice variants have a variety of both 3′ and 5′ acceptor sites for splicing. This is shown in Table 3. There was no preferred major sequence found in the different splice variants in either the 3′ or 5′ splice acceptor sites of the gene.

5′ RACE with Caco2 cells total RNA and two designed primers, based on the N-terminus sequence of PDE9A (r9A and r9Anest, Table 1), revealed the presence of the splice variants PDE9A2 and PDE9A10 in Caco2 cells. No other PDE9A splice variant showing 5′ PDE9A sequences upstream from those already described was found. Therefore, there is no evidence to suggest that the transcriptional start site varies between the different human PDE9A mRNA species.

The sequence comparison between human and mouse genomic sequences of the PDE9A gene (GenBank Accession Nos. AI492065 and NM_000124, respectively) revealed that neither the fifth exon of HsPDE9A nor the new 3b and 4b exons are present in mouse PDE9A gene, although the PDE9A gene structure is essentially conserved (Fig. 2). However the splice structure and the mRNA in mouse appear much simpler as all the ESTs found in the databases are consistent with just a single mRNA similar to the human PDE9A2 variant [called mmPDE9A1A or mmPDE9A2 (GenBank Accession Nos. AF031147 and XM_128503, respectively)]. The expression of the mouse gene has been specially studied

Table 2
Relative intensity of Human Multiple Tissue Expression Array (Clontech) hybridised with a PCR-radiolabelled amplified fragment from fifth PDE9A exon

Tissue	Relative intensity		Tissue	Relative intensity	
Whole brain	+	(581.9)	Oesophagus	+	(459)
Cerebral cortex	+	(166.7)	Stomach	+	(477.6)
Frontal lobe	+	(111.9)	Duodenum	+	(1084.7)
Parietal lobe	+	(219.5)	Jejunum	++	(1738.1)
Occipital lobe	+	(341.3)	Ileum	++	(2056.7)
Temporal lobe	+	(621.9)	Ilocecum	++	(2396.6)
p.g. of cerebral cortex	+	(500)	Appendix	+	(939)
Pons	+	(480.9)	Colon, ascending	++	(2470.5)
Cerebellum left	+	(1526)	Colon, transverse	+++	(5325.4)
Cerebellum right	+	(1303.8)	Colon, descending	++	(2296.6)
Corpus callosum	+	(561.6)	Rectum	++	(2720.4)
Amygdala	+	(680.8)	Bladder	+	(1074.5)
Caudate nucleus	+	(1214)	Uterus	+	(607.8)
Hippocampus	+	(403.6)	Prostate	+++	(8981.8)
Medulla oblongata	+	(393.2)	Testis	+	(116.3)
Putamen	+	(739.9)	Ovary	+	(126.5)
Nucleus accumbens	+	(336.6)	Liver	+	(206.9)
Thalamus	+	(618.6)	Pancreas	+	(211.9)
Heart	+	(422.7)	Adrenal gland	+	(475.1)
Aorta	+	(199.8)	Thyroid gland	+	(322.8)
Atrium, left	+	(307.9)	Salivary gland	+	(620.3)
Atrium, right	+	(198)	Leukaemia, HL-60	–	(0)
Ventricle, left	+	(235.6)	HeLa S3	+	(176.2)
Ventricle, right	+	(333.3)	Leukaemia, K-562	+	(1331.7)
Interventricular septum	+	(353.1)	Leukaemia, MOLT-4	–	(17.3)
Apex of the heart	+	(318.9)	Burkitt's lymphoma Raji	–	(0)
Kidney	++	(1654.6)	Burkitt's lymphoma Daudi	–	(0)
Skeletal muscle	+	(150.4)	Colorectal adenocarcinoma SW480	+	(743)
Spleen	++	(1514.5)	Lung carcinoma A549	+	(70)
Thymus	+	(613.3)	Foetal brain	++	(2729.8)
Peripheral blood leukocyte	+	(48.4)	Foetal heart	+	(327.6)
Lymph node	+	(529.5)	Foetal kidney	++	(2788.3)
Bone marrow	+	(28.8)	Foetal liver	+	(517.1)
Trachea	+	(437.6)	Foetal spleen	+	(955.4)
Lung	+	(321)	Foetal thymus	+	(445.1)
Placenta	+	(432.1)	Foetal lung	+	(796.1)

+++ indicates a relative intensity higher than 5000; ++ indicates a relative intensity between 1500 and 5000; + indicates a relative intensity between 50 and 1500; – indicates a relative intensity lower than 50.

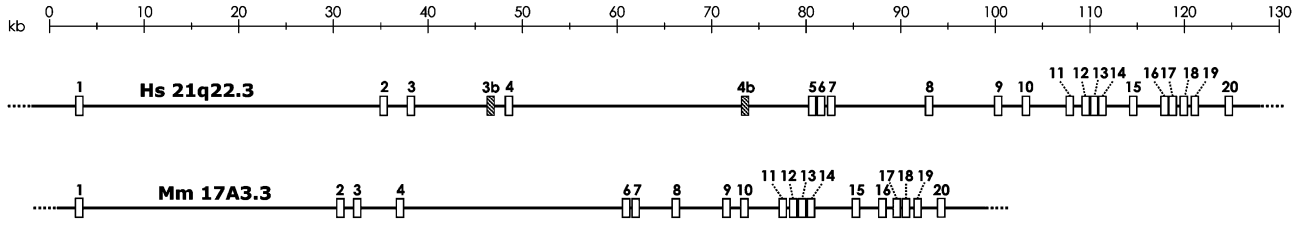

Fig. 2. Schematic representation of human (Hs) and mouse (Mm) PDE9A genes. The genomic structure of the two genes is identical except for exons 3b, 4b, and 5 of human PDE9A, which are not found in the mouse PDE9A gene sequence by BLAST analysis.

in the genome project already published due to its presence in the human chromosome 21. Specific PDE9A mRNA accumulation was found in specific brain regions but also in the gut [22,23], indicating that in these two mammals the function of this protein might be similar.

A feature of some of the splice variants found is that, in order to obtain ORFs with the PDE9A catalytic centre in frame, it is necessary to choose different ATG start codons depending on the splice variant (shown as * in Fig. 3). Alternatively, if the first ATG on exon 1 is taken as the start codon for the protein sequence, a

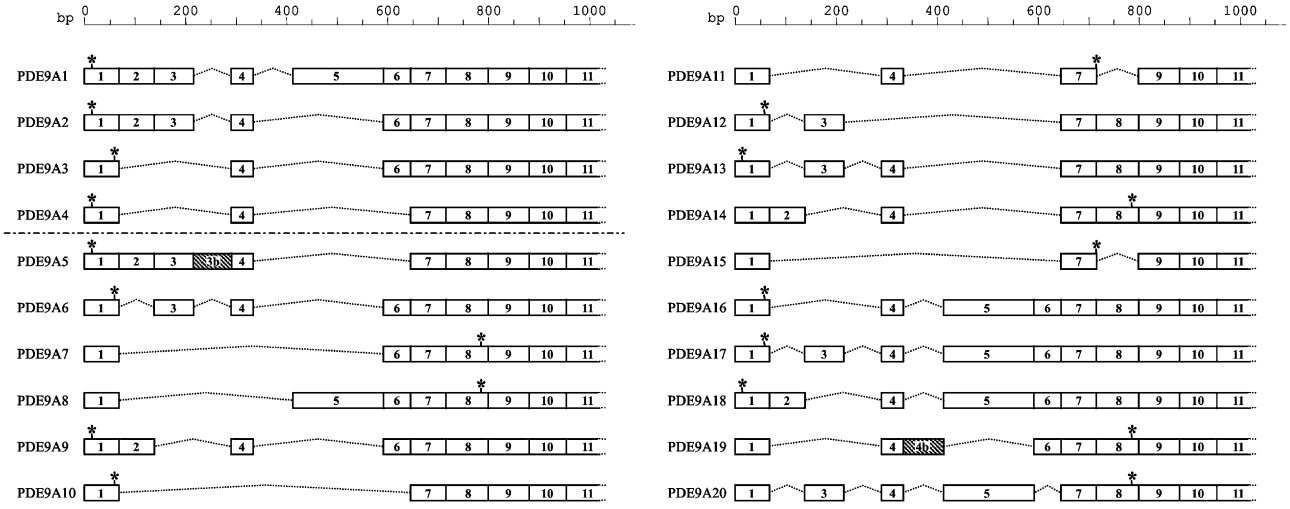

Fig. 3. Schematic representation of the 5′ mRNA structure of the four previously described PDE9A isoforms (PDE9A1–4), together with new PDE9A5–20 splice forms. New exons described in this paper (exons 3b and 4b) are indicated by crossed lines.

Table 3
Intron–exon boundaries of the human PDE9A gene

Exon	Size (bp)	3′ Splice acceptor	5′ Splice acceptor	Genomic position[a]
1	68	ggaaagtACAG	GAAGgtagccc	3063–3174
2	70	tttgcagGTAA	CTCGgtgagtg	35483–35553
3	77	tttccagGAAC	AACGgtaagag	37208–37285
3b	76	cttccagGAAT	CCAGgtgaata	46705–46781
4	43	tttgcagCACT	TCCGgtaaggc	48259–48302
4b	80	tcccccaGCAA	CGGTgtaagta	73846–93925
5	179	tttatagCTGG	CCAGgtaacgg	81061–81240
6	54	tttgtagAGAG	CAAGgtacaga	81361–81415
7	70	ttcctagAGCA	GAATgtgagtg	82647–82717
8	84	tcaccagTGGA	GCAGgtagggt	93067–93151
9	81	tctctagGACC	CAAGgtaagat	100407–100488
10	74	tttctagTACC	TGAGgtaagtg	103281–103355
11	86	cccacagATGC	GCTGgtgagtg	108290–108376
12	104	cccgcagTTCT	CCAGgtgggtc	109619–109723
13	82	aatccagGAGA	ACACgtatgta	110116–110198
14	156	ctcccagGTAC	ACAGgtgtgtg	111374–111530
15	113	tatttagGGAA	CCTGgtgagtg	114672–114785
16	104	ctttcagCTGA	GCAGgtaagag	117479–117583
17	128	cctgcagAGCG	CAAGgtgagta	118318–118446
18	95	ctcccagCTCT	AGAGgtaaaac	119994–120089
19	81	gttccagTTAC	GAAGgtaatgc	121730–121811
20	226	cttccagGAGA	ACGGaaatggg	124571–124797

The nucleotide sequences flanking the 3′ and 5′ splice sites of PDE9A gene are shown. Exon sequences are indicated by upper case letters and intron sequences by lower case letters.

[a] Related to GenBank Accession No. AB017602.

number of splice variants encode shorter sequences with premature stop codons. A similar feature has been previously found in other PDE genes [12,24] and it might represent an additional mechanism for the regulation of PDE activity. It has been shown that this mechanism may generate a non-functional splice variant, as it is the case in the *Drosophila melanogaster Sex-lethal* gene, which generates a non-functional protein in fly males [25]; or a competitor of its function, such as in the non-catalytic domain of the focal adhesion kinase $pp125^{FAK}$, which is autonomously expressed as a separated protein and acts as an inhibitor of the kinase [26]. It is not known if this is the case in any of the human PDE genes studied so far.

Tissue distribution of new splice forms of PDE9A

The splice variants previously described were found in specific cell cultures. It was of interest to observe whether the same variants could be found in specific

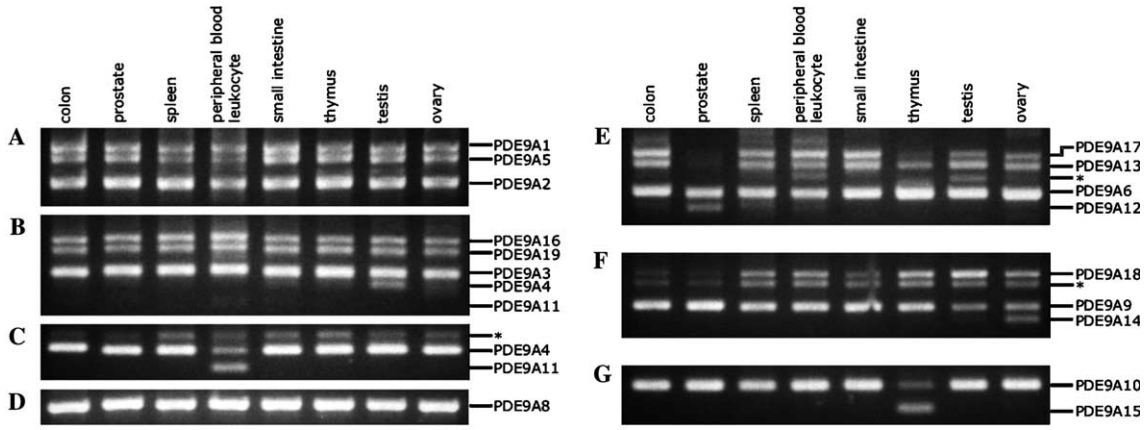

Fig. 4. RT-PCR analysis of the expression of PDE9A isoforms in eight different human tissues. All RT reactions were using the rRT1 primer. Primers used are indicated in Table 1. The results shown are PCR amplifications using: (A) primers d9A2.1-r9A; (B) primers d9A4.1-r9A; (C) nested PCR from PCR d9A4.1-r9A using d9A4.2-r9Anest; (D) primers d9A8-r9A; (E) primers d9A6-r9A; (F) primers d9A9-r9A; and (G) primers d9A10-r9A. The positions of the variants analysed are shown in the figure. Other bands, marked with *, appear to be non-specific PCR products as they did not produce defined results upon sequencing.

human tissues and whether there is any specific distribution between the different tissues. To this end, PCR were done from HeLa, Jurkat, and Caco2 total RNA in order to optimise the PCR conditions of a series of specific PCR primers to show the presence of each different PDE9A splice variant. Using these optimised conditions, PCR were then carried out on eight pools of cDNA from different human tissues with these splice variant specific primers. The results are shown in Fig. 4. It can be observed that most of PDE9A mRNAs are present in all tissues studied. In contrast, we found only PDE9A11 mRNA in peripheral blood leukocytes, PDE9A12 mRNA in prostate, PDE9A14 mRNA in ovary, and PDE9A15 mRNA in thymus. Some other bands (marked as * in Fig. 4) appear to be non-specific PCR products as they did not produce any results upon sequencing.

Two main mechanisms involved in the splicing seem to regulate the gene expression of PDE genes. In some cases, different promoters regulate the transcription of tissue-specific PDE variants, giving different start codons and N-terminal transcripts [19,27–29]. In contrast, other PDE genes have a unique promoter controlling its gene expression and a series of splice mechanisms regulating the domain composition and the start codon of its proteins [30,31]. The human PDE9A gene, like PDE8A [30] and PDE8B [31] genes, appears to have a single transcriptional start site but a number of splice variants in the 5′ region of the gene. The gene has a conserved region of 12 exons containing the catalytic domain of the protein (comprising exons 12–18) that are homologous between human and mouse genes and 10 exons that have multiple splicing possibilities. In some cases (like introns 3b, 4b, and 5) these introns do not have homologous sequences in mouse. The existence of such multiple splice variants appears to be a feature of the human gene. Not all the splice variants are found in all tissues. Although the PCR method used does not allow a quantitative analysis, the presence or absence of specific variants in defined tissues was reproducible. This may indicate the possibility of a regulatory mechanism that in some cases may also give partial protein sequences. This gene appears to keep a first exon in all cases and a multitude of splice variants in the N-terminus of the protein without any clear change in the protein sequences to indicate a defined function of each variant. The main effect of differential splicing in the case of the human PDE9A would then be the production of truncated non-functional proteins.

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