Structure of Two Rat Genes Coding for Closely Related Rolipram-sensitive cAMP Phosphodiesterases

MULTIPLE mRNA VARIANTS ORIGINATE FROM ALTERNATIVE SPLICING AND MULTIPLE START SITES

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The products of two phosphodiesterase (PDE) genes (ratPDE3/IVd and ratPDE4/IVb) are present in the rat Sertoli cell in culture, and their expression is under the control of the gonadotropin follicle-stimulating hormone (Swinnen, J. V., Tsikalas, K. E., and Conti, M. (1991) J. Biol. Chem. 266, 18370–18377). To understand the basis of the sequence heterogeneity found in the 5′-region of the different cDNAs thus far characterized, the structure of the coding region of these two mRNA genes was investigated. Analysis of five ratPDE3/IVd and ratPDE4/IVb genomic clones showed that the coding region of these genes expressed in the Sertoli cell is divided into 11 exons distributed over 35–45 kilobases of genomic DNA. The intron/exon boundaries agreed, with some exceptions, with the established consensus sequences and were located in the same position in the 5′-region of the two genes. Also present were similarities to the exon composition of the Drosophila melanogaster “dunce” gene, the ancestor of these mammalian cAMP PDEs. Multiple AUG codons and short open reading frames were present at the 5′-untranslated end of the ratPDE4/IVb mRNA, but not in the ratPDE3 mRNA. By using polymerase chain reaction amplification or Northern analysis, it was determined that at least two forms of ratPDE3/IVd mRNA are present in rat Sertoli and FRTL-5 thyroid cells, but not in the brain. These mRNA variants are generated by inclusion or removal of an intron sequence that produces a frameshift affecting the position of the initiation AUG codon. Both mRNA species were efficiently translated into cAMP PDE proteins with different molecular masses in a transient transfection assay in COS cells. Polymerase chain reaction amplification demonstrated that heterogeneity of ratPDE4/IVb mRNAs was present in the same location as in the ratPDE3/IVd mRNA. Two ratPDE4/IVb mRNAs with different 5′-ends were expressed in Sertoli and FRTL-5 cells and in the brain. This heterogeneity is caused by the presence of an intron promoter that controls the transcription of this mRNA in Sertoli and FRTL-5 cells, but not in the brain. Upstream exons and additional promoters are probably present and necessary to generate the brain-specific mRNAs. These findings demonstrate that the cAMP-specific PDE genes have complex structure and that cAMP PDE proteins with different amino termini are derived from these genes.

Recent studies on the molecular structure of cyclic-nucleotide phosphodiesterases (PDEs)1 have uncovered an unsuspected complexity of the enzymes that degrade the second messengers cAMP and cGMP. At least 25 different PDE forms have been characterized and have been classified in five distinct families on the basis of their substrate specificity and regulation (1,2). The family of PDEs that hydrolyze cAMP with high affinity (cAMP PDEs), type IV according to a nomenclature recently proposed (2), was originally identified on the basis of the affinity and selectivity for cAMP and specific inhibition by antidepressants like Rolipram and RO 20-1724 (3). Data from our and other laboratories have shown that the expression of these forms is regulated by hormones that act through the cAMP-dependent pathway (reviewed in Ref. 4). In spite of the considerable amount of data available, the definition of the exact biochemical properties of these cAMP PDEs has been elusive. A survey of the literature of the past 10 years has shown that molecular masses ranging from 29 to 80 kDa have been attributed to these forms (3,5–8). In addition, linear and nonlinear kinetics for apparently homogeneous PDE preparations have been observed. An explanation for these conflicting results has come with the cloning of Drosophila melanogaster “dunce” cAMP PDE (9) and, subsequently, of its mammalian homologues (10–13). Analysis of cDNA clones derived from rat testis and brain has indicated that at least four groups of mRNAs encoding closely related proteins are present (10–13). Thus, the presence of more than one kinetically related cAMP PDE form in a given tissue is a likely cause of contrasting physicochemical properties attributed to the cAMP PDEs.

The molecular cloning of the cAMP PDEs has uncovered, however, an additional level of complexity. Northern blot analysis of mRNA retrieved from different tissues demonstrates the presence of transcripts of different sizes for each of the four forms (10–14), with a maximum of five different transcripts for ratPDE1/IVc and five for ratPDE2/IVa found in germ cells (15). Furthermore, three classes of ratPDE2/IVa cDNAs with differ-
ent 5'-ends (RD1, RD2, and RD3) have been retrieved from brain libraries (10), and two variant cDNAs for ratPDE3IVd with different 5'-ends have been detected in the tests (13). Comparison of the ratPDE4IVb sequences retrieved from tests and brain libraries again demonstrates different nucleotide sequences in the 5'-region (12, 14). The presence of multiple mRNA species is compatible with two hypotheses. It is possible that different mRNAs produced by alternative splicing of exons in mRNAs serve to generate cAMP PDE proteins with different amino or carboxyl termini. Regulatory domains affecting catalysis, like the domains binding calmodulin and cGMP, are present at the amino terminus of the calmodulin-regulated PDEs and the cGMP-stimulated PDEs, respectively (16–18). It is then conceivable that the generation of cAMP PDEs with different amino termini is a means to produce enzymes targeted for different regulatory signals. The alternative possibility is that differences at the 5'-end of the mRNAs are the result of the presence of different promoters and different transcription start sites. If this latter hypothesis was correct, different transcription start sites on the gene were responsible for the observed expression and not on the protein product. That the genes encoding cAMP PDE have a complex organization and complex transcription regulation is documented by the characterization of the D. melanogaster gene (19), the probable ancestor of the mammalian cAMP PDEs (9, 19). The gene spans 148 kb of genomic DNA. Three different transcription start sites and alternative splicing generate six different mRNA species (19). In addition, two genes have been mapped within the first intron of this dunce gene (20). Similarly, a Dictostelium discoideum PDE gene has three different start sites that are differentially utilized during development and differentiation of the slime mold (21).

To understand the functional significance of the cAMP PDE mRNA heterogeneity, we have characterized the structure of the two mammalian ratPDE3IVd and ratPDE4IVb cAMP PDE genes expressed in the Sertoli cell and determined the origin of mRNA variants. Evidence consistent with the presence of multiple promoters and alternative splicing indicates a high degree of complexity of these genes in mammals and suggests that different promoters are employed in endocrine cells and in the brain to regulate cAMP PDE expression.

EXPERIMENTAL PROCEDURES

Materials—The Charon 4A rat genomic library was purchased from Clontech; the pWE15 cosm id and a Dash II rat genomic library were from Stratagene. Nylon colony plaque hybridization filters, [γ-32P]ATP, [α-32P]dCTP, and α-32P-dATP were from DuPont NEN. Nylon membranes (Zeta-Probe) were from Bio-Rad. All restriction enzymes were purchased from Boehringer Mannheim, Life Technologies, Inc., or Promega Biotec. Plasmid and λ preparation kits were from Qiagen. The first-strand cDNA synthesis kit and dextran sulfate were from Pharmacia LKB Biotechnology Inc. Oligo(dT)-cellulose T-3 was from Collaborative Research. TheGeneAmp PCR reagent kit and Tq DNA polymerase were from Perkin-Elmer Cetus Instruments. All other chemicals used were analytical grade and purchased from Sigma or Bio-Rad. Oligonucleotides were synthesized using a Pharmacia Gene Assembler.

Isolation and Characterization of Genomic Clones—DNA isolations were carried out using standard methods (22). Three rat genomic libraries (cosmid pW15, λ Dash II, and λ Charon 4A) were screened by plaque hybridization essentially as previously reported for the cDNA libraries. Each cell line L595 was used as the host for plaque lifting. Replica nylon colony plaque hybridization filters were prehybridized for 16 h at 42 °C in 5 x SSC (1 x SSC = 0.15 M NaCl, 0.01 M sodium citrate), 10 x Denhardt's solution (1 x Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.002% bovine serum albumin), 50 mM formamide, 0.5% SDS, and 0.1 ng/ml of worm sperm DNA, and hybridized at 42 °C for 16 h in the presence of 10% dextran sulfate with 106 cpm/ml [32P]-labeled probe (cDNA random primer-labeled or labeled PCR fragments). Filters were washed at temperature in 2 x SSC and 0.5% SDS and at 55 °C in 0.5 x SSC and 0.5% SDS and exposed to Kodak XAR film overnight at −70 °C with intensifying screens. Approximately 500,000 plaque-forming units were screened. Positive clones were purified by repeated screening, and the DNA from resulting clones were purified on ion-exchange columns (Q-Sepharose) according to the manufacturer's protocol. Restriction fragments from these clones were subcloned into the pBluescript KS(+) plasmid vector (Stratagene) for further characterization. Repeated sequencing was performed on both strands with the dideoxy chain termination method of Sanger et al. (23) using Sequenase DNA polymerase according to the protocol supplied by the manufacturer. Alignment of the restriction fragments obtained from each clone was performed by partial digestion and Southern blotting following established procedures (22).

Southern Blot Analysis—Genomic DNA was isolated from Wistar rat liver using the proteinase K digestion method (22). Genomic DNA (30 μg/lane) was restricted with EcoRI, and DNA fragments were fractionated by electrophoresis on 0.8% agarose gel. The DNA was transblotted to nylon membrane (24). Hybridization conditions were the same as those reported above for the library screening.

RNA Preparation—Total RNA from brain tissue or from Sertoli cell cultures was purified by the guanidine thiocyanate extraction method of Chirgwin et al. (25) as previously described (11). Cell extracts were layered on a cushion of 5.5 M CsCl, and RNA was pelleted by centrifugation for 18 h at 100,000 x g in a Beckman SW 40 rotor at room temperature. Poly(A)+ RNA was purified by affinity chromatography on an oligo(dT)-cellulose column (26).

Polymerase Chain Reaction Amplifications—PCRs to generate the probes used in library screenings were performed in a volume of 50 μl containing 500 ng of cDNA, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.01% gelatin, 50 pmol of each amplification primer, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 50 μM of [α-32P]dCTP, and 2.5 units of Tqg polymerase. The reactions were performed for 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C for 25 cycles. At the end of the reaction, products were purified on a Sephadex G-50M column. To retrieve cAMP PDE cDNAs containing additional 5'-sequence, λgt11 tests and Sertoli cell libraries (11, 12) were used as template for PCR amplification. Primers used were ratPDE3IVb-specific oligonucleotide A and N and primers corresponding to the λ sequence flanking the cloning site (forward and reverse primers; Promega Biotec). Amplified fragments were subcloned in the pBluescript KS(+) plasmid and sequenced in both directions.

Reverse Transcriptase PCR—First-strand cDNA was generated using the first-strand cDNA synthesis kit according to the supplied protocol (27). Briefly, 0.2 μg of poly(A)+ RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using random hexadeoxynucleotides as primer. The completed first-strand cDNA reaction product was directly amplified by PCR following the addition of the specific primers and Tqg DNA polymerase. PCR was performed as described in the text.

FIG. 1. Structure of ratPDE4IVb gene derived from two overlapping genomic clones. a, partial restriction map of the genomic clone isolated by screening a λ Dash II library with a PCR fragment containing the 3'-end of cDNA; b, partial restriction map of the genomic clone isolated by screening a λ Dash II library with a PCR fragment containing the 5'-end of cDNA; c, scheme of the gene structure (exons are represented by filled boxes and are numbered; introns are represented by lines and are lettered). The sizes of the EcoRI fragments that contain the exons and that hybridize to a full-length cDNA are reported below the gene structure. The hatched box corresponds to the 5'-coding sequence expressed in Sertoli and FRTL-5 cell mRNAs. Although the presence of exon 1a is inferred by PCR analysis of the mRNA, its position in the gene has not been mapped.
scribed above. An aliquot of PCR was analyzed on a 3% NuSieve agarose gel (FMC Bioproducts) in 0.5 M Tris borate and 0.002 M EDTA and stained with ethidium bromide.

**Primer Extension**—Primer extension was performed according to Sambrook et al. (22). The oligonucleotides used (see Fig. 9) were end-labeled with T4 polynucleotide kinase and [y-32P]ATP. The radioactive probe was hybridized to 20 μg of Sertoli cell RNA overnight at 30 °C in 30 μl of buffer containing 40 μM Pipes, pH 6.8, 0.4 M NaCl, 1 μM EDTA, and 80% formamide. The hybridization mixture was then precipitated, and the RNA was resuspended in 50 μl of reverse transcription buffer (50 μM Tris-HCl, pH 7.5, 60 μM KCl, 10 mM MgCl2, 1 μM each dNTP, 1 μM dithiothreitol, 1 μl of RNasin, 50 μg/ml actinomycin D; 50 units of avian myeloblastosis virus reverse transcriptase was added, and the incubation was performed for 90 min at 42 °C. At the end of this incubation, a template RNA was removed by digestion with DNase-free RNase, and the reaction product was extracted with phenol/chloroform and then ethanol-precipitated. The resulting pellet was resuspended in formamide loading buffer (80% formamide, 10 μM EDTA, 1 μg/ml xylene cyanol, 1 μg/ml bromophen blue) and analyzed on a 6% denaturing urea-polyacrylamide gel.

**Transfection and Protein Detection by Western Analysis**—Transient transfection of COS or MA-10 cells was performed using the CaPO4 DNA method (28) following a procedure previously reported (29). The pCMV-ratPDE3.1 construct has been previously described (29). The pCMV-ratPDE3.2 construct was obtained by subcloning the ratPDE3.2 cDNA (13) into the EcoRI restriction site of the pCMV polylinker. The pCMV-ratPDE3.2 construct has been previously described (29). The pCMV-ratPDE3.1 construct has been previously described (29). The pCMV-ratPDE3.2 construct was obtained by subcloning the ratPDE3.2 cDNA (13) into the EcoRI restriction site of the pCMV polylinker. The resulting plasmid was sequenced using a DNA fragment prepared by amplifying a region of 155 bp containing the entire open reading frame (13), PCR fragment probe was hybridized to 20 pg of Sertoli cell RNA overnight at 30 °C in 30 μl of buffer containing 40 μM Pipes, pH 6.8, 0.4 M NaCl, 1 μM EDTA, and 80% formamide. The hybridization mixture was then precipitated, and the RNA was resuspended in 50 μl of reverse transcription buffer (50 μM Tris-HCl, pH 7.5, 60 μM KCl, 10 mM MgCl2, 1 μM each dNTP, 1 μM dithiothreitol, 1 μl of RNasin, 50 μg/ml actinomycin D; 50 units of avian myeloblastosis virus reverse transcriptase was added, and the incubation was performed for 90 min at 42 °C. At the end of this incubation, a template RNA was removed by digestion with DNase-free RNase, and the reaction product was extracted with phenol/chloroform and then ethanol-precipitated. The resulting pellet was resuspended in formamide loading buffer (80% formamide, 10 μM EDTA, 1 μg/ml xylene cyanol, 1 μg/ml bromophen blue) and analyzed on a 6% denaturing urea-polyacrylamide gel.

**RESULTS**

**Organization of ratPDE4/IV and ratPDE3/IV Genes**—A cDNA containing the major open reading frame of ratPDE4/IV (11) was used to screen a genomic library prepared in cosmID5. One positive clone (pWE15-ratPDE4.1), containing an insert of ~25 kb, was identified and characterized by digestion and Southern blot analysis (Fig. 1). The fragments containing exon sequences were subcloned into the pBluescript KS(+) plasmid and sequenced using exon-specific primers. Comparison of the genomic fragment sequences with the corresponding cDNA sequence made it possible to map the positions of the exons in the gene (Fig. 1). To obtain the 3'-portion of this gene, a library constructed in λ DASH II was screened using a DNA fragment prepared by amplifying a region of 155 bp at the 3'-end of cDNA (corresponding to bp +1545 to +1699 of the ratPDE4/IV cDNA; oligonucleotides P and O in Fig. 2) as a probe. From this screening, four positive clones were retrieved, and one (λ DASH II-ratPDE4.2) was further characterized. EcoRI digestion of this clone revealed the presence of six genomic fragments, and each fragment was subcloned, sequenced, and mapped in the PDE4/IV gene (Fig. 1). Identical sequences were found in fragments of 4.3 kb of a λ DASH II-ratPDE4.2 and 5.2 kb of pWE15-ratPDE4.1, indicating that these clones overlap in this region. Comparison of the cDNA sequence with the genomic sequence allowed a tentative assignment of the intron/exon boundaries (Table I). Most junctions followed the consensus proposed by Mount (30) and the GT-AG rules (31). The nucleotide sequences of the exons and intron/exon junctions are reported in Fig. 2. This portion of the ratPDE4/IV gene spans at least 35 kb, and it is arranged in 11 exons and 10 introns. The exons are located on five distinct EcoRI restriction fragments of 3.7 kb (including exons 1 and 2), 4.9 kb (exon 3), 5.2 kb (exons 4–7), 3.5 kb (exons 8–10), and 2.1 kb (exon 11). With the exception of the first and last exon, the lengths of the exons range from 93 to 183 bp, whereas the lengths of the introns, evaluated by restriction analysis of the genomic clones or PCR amplification, range from 0.5 to 10 kb.

To obtain all the coding fragments of the ratPDE4/IV gene, λ Charon A and λ DASH II libraries were screened with the following probes: a ratPDE3.1 cDNA containing the entire open reading frame (13), PCR fragment A/B (obtained by amplifying the region corresponding to bp −3 to +260 on cDNA; see Fig. 4),
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The nucleotide sequence of the ratPDE4/TVb gene. The exon sequences are shown in upper-case letters; the intron sequences are shown in lower-case letters. The location of the 3' splice site of intron A is marked by a downward arrow. This intron is spliced out in the brain mRNA, but functions as a coding exon in the Sertoli cell mRNA. The deduced amino acid sequence is shown below the nucleotide sequence using the single-letter amino acid code. Nucleotides are numbered from the first in-frame translation initiation codon (marked in boldface), and the numbers are reported on the left above the nucleotide sequence. Putative sites of transcription initiation are marked by white downward arrows.

Fig. 2. Nucleotide sequence of the ratPDE4/TVb gene. The exon sequences are shown in upper-case letters; the intron sequences are shown in lower-case letters. The location of the 3' splice site of intron A is marked by a downward arrow. This intron is spliced out in the brain mRNA, but functions as a coding exon in the Sertoli cell mRNA. The deduced amino acid sequence is shown below the nucleotide sequence using the single-letter amino acid code. Nucleotides are numbered from the first in-frame ATG codon (marked in boldface), and the numbers are reported on the left above the DNA sequence. The oligonucleotide primers used for PCR analyses are also indicated by arrows above the nucleotide sequence. Putative sites of transcription initiation are marked by white downward arrows.

and PCR fragment I/E (amplification product corresponding to bp 261–335 on the ratPDE3.1 cDNA; see Fig. 4). The genomic clones isolated were further characterized by the same strategy indicated for the ratPDE4/TVb gene. Clone Charon 4A-ratPDE3.1, retrieved by screening the library with the full-length cDNA, covers most of the gene except the 5'-end. Clone Dash II-ratPDE3.1, obtained by screening the library with PCR fragment I/E, overlapped with the Charon 4A-ratPDE3.1 clone. No overlap was found with clone Dash II-ratPDE3.2 (Fig. 3b), and one gap was present in the intron region between exons 2 and 3. The restriction map of the genomic clones isolated as well as the exons they include is shown in Fig. 3, whereas the nucleotide sequence of the ratPDE3/IVd intron/exon boundaries are reported in Fig. 4. Intron/exon boundaries of ratPDE3/IVd contain the consensus 5'- and 3'-splice sequences (Table II) and were located in the same position as in ratPDE4/TVb (Fig. 2).
EcoRI fragments: 6.4 kb (exons 1 and 2), 2.6 kb (exon 3), 4.2 kb

AUG codon previously assigned as the first in-frame AUG

result of relatively short (100-300 bp) and are contained in five distinct cDNAs were com-

bridging to ratPDE3IVd and ratPDE4IVb cDNAs were com-

quences showed similar exon composition (Fig. 5). The ratPDE3IVd introns reach 9 kb in length, and the exons are

are represented by filled boxes and are numbered; introns are represented by lines and are lettered). The sizes of the EcoRI fragments that contain exons and that therefore hybridize to cDNA are reported below the gene diagram. The presence of one or more additional exons, as inferred by amplification data, is depicted as a filled box and is identified as 1a?. The location of these putative exons in the gene has not been mapped.

5). Alignment of the splice junction sequences of ratPDE3IVd and ratPDE4IVb with the D. melanogaster dunce exon sequences showed similar exon composition (Fig. 5). The ratPDE3IVd introns reach 9 kb in length, and the exons are relatively short (100-300 bp) and are contained in five distinct EcoRI fragments: 6.4 kb (exons 1 and 2), 2.6 kb (exon 3), 4.2 kb (exons 4-6), 8.6 kb (exons 7-9), and 2.8 kb (exons 10 and 11).

Genomic Southern Blot Analysis—Wistar rat genomic DNA digested with EcoRI was hybridized to ratPDE3IVd and ratPDE4IVb cDNAs. The sizes of the genomic fragments hybridizing to ratPDE3IVd and ratPDE4IVb cDNAs were comparable to those predicted from the restriction map of the corresponding genomic clones (Fig. 6). However, the doublet in the 2.8-kb region of ratPDE3IVd and in the 3- and 5-kb regions of ratPDE4IVb/IVb inferred from the restriction analysis of the genomic clones could not be completely resolved on the Southern genomic blot using the cDNAs as probe. Hybridization with exon-specific probes confirmed the presence of multiple fragments (data not shown).

Characterization of Different ratPDE3IVd and ratPDE4IVb mRNAs—Since none of the cDNAs encoding Sertoli cell ratPDE4IVb (11) had an in-frame termination codon upstream from the first AUG codon, available cDNA libraries were used as template in a PCR to obtain cDNAs with more 5'-upstream sequence (see “Experimental Procedures”). Following this strategy, a clone with 100 base pairs of additional 5'-sequence of the ratPDE4IVb cDNA was isolated. This includes termination codons in all three reading frames, demonstrating that the AUG codon previously assigned as the first in-frame AUG codon of the ratPDE4IVb mRNA expressed in Sertoli cells was correctly identified (see Fig. 2).

As previously reported, ratPDE4IVb cDNAs with different 5'-ends were retrieved from testis and brain libraries (11, 12, 14). Since the brain-specific sequence was not present in the ratPDE4IVb gene sequence obtained, reverse transcriptase PCR was performed with brain and Sertoli cell poly(A)+ RNAs. An antisense primer (oligonucleotide A) common to both tissue sequences was used. The sense primers were specific for the 5'-cDNA sequence of rat brain (oligonucleotide W) (12) and Sertoli cell sequence (oligonucleotide M). Amplification products were present only when the primers specific for each tissue were used, and the sizes of these fragments were identical to those predicted by the corresponding cDNAs (Fig. 7). Furthermore, oligonucleotide W, specific for the brain mRNA, did not hybridize either to the 3.7-kb EcoRI fragment containing the putative start site of ratPDE4IVb or to a 4-kb EcoRI fragment located upstream (data not shown). This suggests that the sequence present in the brain mRNA is, in the gene, more than 5 kb upstream from the Sertoli cell exon and that the start site of the Sertoli cell mRNA derives from intron sequences.

Sequencing of the 4.0-kb XbaI fragment of the ratPDE3IVd gene containing exons 1 and 2 confirmed that the 87-bp sequence found in ratPDE3.1 cDNA is not a cloning artifact, but is rather due to unspliced intron sequence. The presence of the splicing variant mRNAs originating from this exon 1/exon 2 boundary was further investigated by reverse transcriptase PCR. Poly(A)+ RNA was prepared from Sertoli and FRTL-5 thyroid cell cultures and from rat heart and brain tissues and used as template for reverse transcriptase PCR using the two oligonucleotides (B and A) flanking the putative intron as primers. Two products of 265 and 179 bp were amplified from the Sertoli cell and FRTL-5 mRNAs. These sizes correspond to those expected when the intron is spliced in or out (Fig. 8) and were identical to the products of amplification of the ratPDE3.1 and ratPDE3.2 cDNAs. In contrast, very little of the variant without the intron could be amplified from the heart mRNA. In spite of the fact that ratPDE3IVd mRNA transcripts have been detected by Northern analysis of brain mRNA (13), the two primers could not amplify the proper fragments in any of the four different brain mRNA preparations used. That the ratPDE3IVd mRNA is present in these brain mRNA preparations was demonstrated by the fact that primers in exons 3 and 4 of ratPDE3IVd amplify fragments of the correct size (data not shown). This latter finding is compatible with the hypothesis that additional splice variants at the boundary of exons 1 and 2 of ratPDE3IVd are expressed in the brain.

To determine whether the intron sequence is present only in high molecular mass unprocessed RNAs or in mature mRNA, a probe generated by PCR and corresponding exclusively to the intron sequence was labeled and used for Northern blot hybridization of Sertoli cell mRNA. A transcript of 6.7 kb hybridized to this probe (data not shown). This is the same size as the transcript that hybridizes to a cDNA containing the complete coding region (13), therefore confirming that this intron sequence ap-

Fig. 3. Structure of ratPDE3IVd gene derived from three genomic clones. a, EcoRI restriction map of the genomic clone isolated by screening the A Charon 4A library with the full-length cDNA; b, partial restriction map of the genomic clone isolated by screening the A Dash II library with a PCR fragment amplified with oligonucleotides A/B of Fig. 4; c, partial restriction map of the genomic clone isolated by screening the A Dash II library with a PCR fragment amplified with oligonucleotides B/I of Fig. 4; d, diagram of the gene structure (exons are represented by filled boxes and are numbered; introns are represented by lines and are lettered). The sizes of the EcoRI fragments that contain exons and that therefore hybridize to cDNA are reported below the gene diagram. The presence of one or more additional exons, as inferred by amplification data, is depicted as a filled box and is identified as 1a?. The location of these putative exons in the gene has not been mapped.

Multiple mRNAs from the cAMP PDE Genes
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Mapping of ratPDE3/IVd and ratPDE4/IVb Transcription Start Sites in Sertoli Cells—In a preliminary attempt to determine the transcription initiation sites active in the Sertoli cell, primer extension and reverse transcriptase PCR experiments were performed using mRNA derived from Sertoli cells incubated for 24 h in the presence of 1 μM dibutyryl cAMP. This incubation was included to increase the relative abundance of the two mRNAs (13). Primer extension was performed with oligonucleotides K and C, which are complementary to the ratPDE4/IVb sequence (see Fig. 2). In the three independent experiments performed, several extension products were observed with both primers. Extension with oligonucleotide K revealed major products of 615 and 550 bp (Fig. 9) and one or two faint bands of 750 and 840 bp, respectively. Two major (194- and 249-bp) and two minor (480- and 340-bp) products were observed when oligonucleotide C was used. The predominant extension product of 615 bp using oligonucleotide K corresponds to the product of 249 bp extended with oligonucleotide C and terminates between bases −449 and −439. The CAP sequence TCAGTG'IT is present in this location and is 41 bp downstream from a sequence homologous to a TATA box (32). Sequence TCAGTG'IT is present in this location and is 41 bp downstream from a sequence homologous to a TATA box (32).
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Exon sequences are indicated by upper-case letters, and intron sequences by lower-case letters. Exon 1a is a putative exon inferred by the PCR amplification of the brain mRNA. Location of the exons is based on the numbering of the sequence reported in Fig. 4, where the A residue of the first ATG codon is base 1.

| Table II | Intron exon boundaries in ratPDE3 |
|----------|----------------------------------|

| No. | Location (DNA) | Length | 5'-Splice | Intron | 3'-Splice | Codon phase |
|-----|----------------|--------|-----------|--------|-----------|-------------|
| 1a  | ?              | ?      | bp        | A      | ttcgcaGAGGAG | 1           |
| 1b  | 1              | 90     | GGC GGGgtacgg | ← A →  |
| 2   | 139            | 111    | AACAAGgtagc | ← B →  |
| 3   | 250            | 94     | TCTTAGgtgaga | ← C →  |
| 4   | 344            | 173    | GCAAGgtgagc | ← D →  |
| 5   | 517            | 99     | TTTCAAGtgatcc | ← E →  |
| 6   | 616            | 165    | tTGGAgtgagc | ← F →  |
| 7   | 781            | 100    | A?CAACtgtagct | ← G →  |
| 8   | 881            | 156    | GACATTgtgagtc | ← H →  |
| 9   | 1037           | 122    | ATCCAGtgatataa | ← I →  |
| 10  | 1150           | 183    | TCACGtgatatt | ← J →  |
| 11  | 1342           | >400   |           |        |           | 0           |

Fig. 5. Comparison of deduced amino acid sequences of cAMP PDEs from Sertoli cells and Drosophila duncPDE. The amino acid sequences of the major open reading frames deduced from ratPDE3/Vd, ratPDE4/Vb, and Drosophila (Dro) PDE type I mRNAs are aligned. The putative initiation methionine is derived from the first in-frame ATG codon. Exons correspond to boxes around the amino acid sequence. Dashes indicates gaps inserted to maintain the alignment of the sequences.

Different mRNA Variants Are Translated into Proteins of Different Mass—To test whether the different mRNA variants are translated into different protein products, ratPDE3.1, ratPDE3.2, and ratPDE4 cDNAs were expressed in COS cells. The expression of Sertoli cell ratPDE4/M has been previously reported (14). Both ratPDE3/Vd cDNA variants produced proteins with catalytic activity and immunoreactive bands recognized by the K116 antibody. The ratPDE3.1 cDNA directed synthesis of proteins of 72-74 kDa and a doublet of 67-68 kDa (Fig. 11). The ratPDE3.2 cDNA expression led to the appearance of only the 67-68 kDa band doublet. The migration
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DISCUSSION

The data reported provide evidence that multiple cAMP PDE mRNAs are derived from the ratPDE3/IVd and ratPDE4/IVb genes and are expressed in a tissue-specific manner in the brain and in endocrine cells. These mRNA variants are derived from different transcription initiation sites and alternative exon usage and/or differential splicing of introns. Thus, intrinsic regulatory mechanisms serve to control the expression and function of the cAMP PDE genes in a tissue-specific manner.

The ratPDE3/IVd and ratPDE4/IVb mRNAs expressed in the Sertoli cell derive from the assembly of 11 coding exons. Exons 5–10 of the two genes encode the catalytic region of the protein. This assumption is based on deletion mutations performed on ratPDE3md cDNA (33) that demonstrated that removal of sequences in exons 14 and 11 produces a protein still catalytically active. Site-directed mutagenesis of ratPDE3md also shows that residues crucial for structure and function of the catalytic site are located in exons 7 and 8 (33). These are among the most conserved exons. The sequences of exons 7-9 and the location of the intron splicing are similar to those of exons 13 and 14 of the P-cGMP-specific retina PDE (34), suggesting that structural homologies are present between different families of PDE genes. In addition to the five exons corresponding to the catalytic domain, it was also noted that exon 2, adjacent to the alternative splicing site, contains a domain highly conserved in all rat and human CAMP PDE sequences available (10–13, 35, 36). Exon 4 contains a highly charged sequence (KEKEKKKR) in ratPDE3/IVd and ratPDE4/IVb. This motif is absent in the Drosophila dunce PDE and ratPDE2/IVa, where an insertion of 8–20 amino acids has occurred.

It is likely that similar intron/exon distribution is present in the other two cAMP PDE genes present in rodents (ratPDE1/IVc and ratPDE2/IVa). Davis et al. (10) have retrieved cDNAs with multiple 5'-ends from rat brain libraries. One of these
Multiple mRNAs from the cAMP PDE Genes

![Image of a figure with diagrams and tables]

**Fig. 9.** Primer extension analysis of RNA from cultured Sertoli cells. Rat Sertoli cell total RNA (30 µg) was hybridized to 5'-end-labeled oligonucleotides K and C (ratPDE4-specific) and D (ratPDE3-specific) and extended with avian myeloblastosis virus reverse transcriptase. The primer-extended products were separated on a denaturing polyacrylamide gel as described under “Experimental Procedures.” The major extension products for each specific primer are indicated by the arrows, followed by its estimated length. The sizes of the extended products were determined relative to the migration of λ phage markers.

Extension experiments were repeated three to four times with similar results.

**Fig. 10.** Identification of 5’-sequence of ratPDE4/IVb gene present in Sertoli cell mRNA by reverse transcriptase PCR. Poly(A)+ RNA from Sertoli cell cultures (1 µg) was reverse-transcribed into cDNA and subjected to PCR. PCR products were analyzed by electrophoresis on a 3% agarose gel with ethidium bromide staining. The experiment was repeated three times with similar results.

The primer-extended products for each specific primer are indicated by the arrows, followed by its estimated length. The sizes of the extended products were determined relative to the migration of λ phage markers.

Extension experiments were repeated three to four times with similar results.

**Fig. 11.** Western blot analysis of recombinant ratPDE3/IVd PDE variants expressed in COS cells. Cells were transfected with pCMV-ratPDE3.1 and pCMV-ratPDE3.2 expression vectors. The ratPDE3.2 cDNA does not contain the first intron sequence, whereas ratPDE3.1 still contains the intron. After 48 h of culture, cells were harvested, and the PDE activity was immunoprecipitated using a polyclonal antibody. The characteristics of the antibody and the immunoprecipitation procedure have been previously reported (14, 33). The proteins absorbed were eluted from the antibody with SDS and analyzed by SDS-polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, the blot was incubated with the same antibody and with radioactive protein A. Immunoprecipitated samples from cells processed for transfection with plasmid without the insert are also shown (Mock).

**Result:** RatPDE3/IVd mRNA should be translated at a slower rate than ratPDE3/IVb. According to the mRNA scanning hypothesis (37), ratPDE4/IVb mRNA should be translated at a slower rate than ratPDE3/IVd.

It is striking that the beginning of the heterogeneity of the ratPDE3/IVd mRNA was found at the exact same location as the tissue-specific ratPDE4/IVb mRNA alternative splicing.

cDNAs, RD3, has a region of 99 base pairs removed. Since the boundaries of the sequence missing in RD3 are in a location identical to that of exon 5 of ratPDE3/IVd and ratPDE4/IVb, this region probably corresponds to an exon also in the ratPDE2/IVa gene. The significance of the removal of this exon is unknown, but it again points to an intricate splicing pattern of this cAMP PDE gene. Several intron/exon boundaries are located in the same position in the D. melanogaster gene and in ratPDE3/IVd and ratPDE4/IVb (19). One major difference between the structures of the ratPDE3/IVd and ratPDE4/IVb genes (this report) and of the Drosophila dunce gene is that additional noncoding and coding exons are present at the 5'-end of the latter gene. Exon 2 of ratPDE3/IVd and ratPDE4/IVb is homologous to exon 6 of the Drosophila dunce gene.

We should emphasize that the exon composition that we report is deduced from the characterization of the mRNAs expressed in the Sertoli cell. On the basis of our preliminary findings and the homology to the Drosophila dunce gene, it is likely that the ratPDE3 and ratPDE4 mRNAs expressed in the brain contain additional 5'-exons (Fig. 12).

A distinctive feature of the ratPDE3/IVd and ratPDE4/IVb mRNAs derived from the Sertoli cell is the 5'-untranslated sequence. Unlike what is observed for ratPDE4/IVb, the ratPDE3/IVd 5'-untranslated region is very GC-rich (70%), suggesting a complex secondary structure of this mRNA. Furthermore, whereas the ratPDE3/IVd 5'-untranslated sequence contains only one AUG codon, the ratPDE4/IVb 5'-untranslated sequence has nine AUG codons in the three reading frames. These AUG codons are all followed by stop codons determining the presence of short open reading frames. It has been proposed that the presence of these "mini-open reading frames" and of AUG-burdened 5'-untranslated sequence may substantially reduce the translatability of the mRNA (37). According to the mRNA scanning hypothesis (37), ratPDE4/IVb mRNA should be translated at a slower rate than ratPDE3/IVd.

It is striking that the beginning of the heterogeneity of the ratPDE3/IVd mRNA was found at the exact same location as the tissue-specific ratPDE4/IVb mRNA alternative splicing
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![Diagram](image)

**Fig. 12. Proposed alternative splicing model for generation of different ratPDE3/Vd and ratPDE3/Vd mRNA variants present in Sertoli cell and in brain.** The structures of the two genes are as shown in Figs. 1 and 3. Exons are indicated by filled boxes, and introns by connecting lines. The hatched box in the ratPDE3/Vb Sertoli cell mRNA represents the coding region derived from the intron start site, the region absent in the brain mRNA. The stippled box in the Sertoli cell type I mRNA represents the coding sequence derived from the unspliced intron. The broken box marked IAF in the ratPDE3/Vd gene represents the upstream exons utilized in the brain mRNA. Its presence is inferred by PCR amplification data on brain mRNA and by comparison with the Drosophila dunce gene.

Boundary. This, together with the fact that the boundary of exons 1 and 2 is highly conserved in the two genes, is an indication that evolutionary pressure has maintained the intron sequence and the splicing variants. If splicing mRNA variants are found at the same boundary in other species, it would be further proof that this splicing is conserved and physiologically relevant.

PCR analysis of mRNAs from Sertoli and FRTL-5 cells shows that a subclass of ratPDE3/Vd transcripts contains an intron sequence that is probably spliced out at a rate much slower than the other introns. That this intron splicing is delayed is indicated by the finding that oligonucleotide A is able to amplify the mRNA containing this intron. This oligonucleotide was designed across the splice site between exons 2 and 3. Thus, if intron B had not been removed, amplification would not have occurred. That a substantial portion of the mature mRNA contains the intron sequence and that this intron is removed late are also indicated by the finding that a probe corresponding to the intron sequence hybridizes to the major Sertoli cell transcript of 6.7 kb. Since, in our experiments, nuclear RNA was not separated from cytoplasmic mRNA, it is not known whether the intron sequence is present only in nuclear RNA or also in cytoplasmic RNA. Pending further studies to define this point, the splicing of this intron could be a mechanism by which mRNA translocation to the cytoplasm and/or translation is delayed. This would be similar to what is shown for other mRNAs in which unspliced intron sequences serve to control the rate of translation (38, 39).

It is also possible that the presence of the unspliced intron in the ratPDE3/Vd mRNA serves to generate proteins with different amino termini since it is predicted that two different AUG codons are used in the two type I and II mRNAs (see Fig. 12). That both ratPDE3/Vd mRNAs appear to be translated into functional proteins is documented by the transient transfection experiments. Expression of the cDNA lacking the intron (ratPDE3.2) produces a doublet of 67–68 kDa and an active PDE. The expression of a cDNA containing the intron sequence (ratPDE3.1) produces instead two polypeptides, one of 72–74 kDa and one of 67–68 kDa. Thus, the higher molecular mass polypeptide most likely originates from the mRNA with the intron sequence (type I mRNA). The cause of the appearance of the lower 67–68-kDa doublet after transfection of the cDNA containing the intron could be 2-fold. Some splicing of the intron might occur during the transient transfection, and both mRNAs would be translated into proteins of different molecular mass. Alternatively, both AUG codons of the type I mRNA might be used as the result of leaky scanning (37). Since the endogenous Sertoli cell ratPDE3/Vd protein migrates as a doublet with a molecular mass of 67–68 kDa, we can conclude that this originates from an mRNA in which the intron has been removed (type II mRNA). In addition, unlike what is seen with transient transfection, stable transfection of the ratPDE3.1 cDNA produces only a protein of 67–68 kDa. The whole of these data would indicate that the type II mRNA is the form translated in vivo and that the presence of the intron has no bearing on the protein sequence in the Sertoli cell. However, amplification of the rat heart mRNA demonstrates that most of the RNA contains the intron sequence (type I mRNA), and a 72–74-kDa immunoreactive protein was detected by Western analysis of extracts from this tissue. These latter findings are consistent with the hypothesis that, unlike what is observed in the Sertoli cell, ratPDE3/Vd expressed in the heart is derived from the type I mRNA and that removal of intron A might be tissue-dependent.

Another interesting result is the fact that oligonucleotides corresponding to exons 1 and 2 of ratPDE3/Vd can amplify a product from the testis, thyroid cells, and heart, but not from brain mRNA, whereas oligonucleotides in exons 3 and 4 and ratPDE4/Vb-specific oligonucleotides are able to amplify a band of the correct size from brain mRNA. Since previous Northern blot analyses have shown that ratPDE3/Vd mRNAs are abundant in the brain (13), it has to be postulated that an additional splice variant in the region between exons 1 and 2 is present in the brain (see Fig. 12). This indicates that an even more intricate regulation of exon splicing occurs in the ratPDE3/Vd gene in the brain and opens the possibility that additional promoters might be present, as we have suggested for ratPDE4/Vb. Experiments are underway to clarify this point.

Our data show that transcription of these genes in the Sertoli cell is directed by intron promoters and suggest that the expression of these CAMP PDEs in the brain is directed by additional upstream promoters. Once the structure of the genomic regions active in the brain is characterized, it will be possible to confirm whether these promoters are indeed brain-specific. In view of our finding that the expression of the cAMP PDEs is hormone- and camp-dependent in endocrine cells (13, 14), it will be important to define whether camp regulation of transcription of these PDE genes is dependent on one or more promoters, whether different promoter usage causes differences in camp sensitivity, and whether hormones can dictate which promoter is used. Certainly, the expression of these CAMP PDEs appears to be very finely regulated, implying that these enzymes play a crucial role in the function of the brain and of endocrine organs.

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2 J. V. Swinnen and M. Conti, unpublished observation.
4 S. Iona and M. Conti, manuscript in preparation.
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