aberrant activation to generate such a constitutive proliferation response (at least in the absence of other events). The generation of suitable protein kinase C cDNA constructs should make it possible to determine whether the expression of this kinase is abnormal in any human neoplasias.

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Multiple, Distinct Forms of Bovine and Human Protein Kinase C Suggest Diversity in Cellular Signaling Pathways

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A new family of protein kinase C–related genes has been identified in bovine, human, and rat genomes. The alpha-, beta-, and gamma-type protein kinase sequences are highly homologous, include a kinase domain, and potential calcium-binding sites, and they contain interspersed variable regions. The corresponding genes are located on distinct human chromosomes; the possibility of even greater genetic complexity of this gene family is suggested by Northern and Southern hybridization analyses.

ACTIVATION OF THE PHORBOL ESTER RECEPTOR PROTEIN kinase C (PKC) by calcium ions and the second messenger diacylglycerol is thought to play a central role in the induction of cellular responses by a variety of ligand-receptor systems (*1–3*) and in regulation of cellular responsiveness to external stimuli (*4, 5*). Structural characterization of bovine PKC (bPKC) by partial peptide sequencing and complementary DNA (cDNA) cloning has now provided information that facilitates the analysis of its multiple activities, including diacylglycerol and calcium binding, membrane association, serine and threonine residue phosphorylation, and substrate recognition (*6*). It remains unclear, however, how a single molecule can mediate and control a variety of diverse signaling pathways as has been suggested for PKC (*1–3*). We report here, for the first time, the existence of two bovine and human genes that encode polypeptide sequences with remarkable homology to PKC. We find homologs of bPKC-related messenger RNA's (mRNA's) in human and rat tissues and demonstrate that corresponding genes map to distinct chromosomal loci of the human genome. Southern hybridization analysis (*7*) suggests the existence of additional members of this newly identified PKC gene family.

Screening of a bovine brain cDNA library with a pool of synthetic oligonucleotide probes that had been designed on the basis of

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bovine brain protein kinase C tryptic peptide sequences (*6*) (Fig. 1A) reproducibly generated hybridization signals of variable intensity. After confirming that the strongly hybridizing clones represented bPKC cDNA's, we also characterized the second class of hybridization positives. Southern hybridization analysis (*7*) of Eco RI–digested clones revealed that one of the probes (probe 5) hybridized with approximately equal efficiency to clones λbPKC15 and -19 (Fig. 1B), each about five times lower than the level of hybridization detected with the λbPKC21 control (Fig. 1B). Interestingly, probe 4 hybridized to λbPKC19 but not to λbPKC15 and -21. These results prompted us to characterize in detail the clones λbPKC15 and λbPKC19. Our initial sequence analysis demonstrated that these clones contained cDNA's that were closely related but distinct from protein kinase C (clone λbPKC21). Comparison of the cDNA nucleotide sequences with the sequences of oligonucleotide probes readily explained the differences in hybridization signals observed in our initial screening and subsequent Southern analysis (Fig. 1A). The 26-nucleotide perfect match between probe 5 and the corresponding sequence in λbPKC21 is interrupted twice in λbPKC15 and -19; only λbPKC19 shows a 34-nucleotide stretch with only two mismatches with probe 4, whereas the larger number of mismatches in λbPKC15 and λbPKC21 prevented hybridization to this oligonucleotide.

Sequences related to protein kinase C. On the basis of these findings, we designated the clone corresponding to the isolated protein sequence as protein kinase C–alpha type (PKCα) and the related cDNA sequences as PKCβ and PKCγ. Further screening of the bovine brain cDNA library with cDNA inserts bPKCβ15 and bPKCγ19, in addition to specific synthetic oligonucleotide probes (see legend to Fig. 2), led to the isolation of the sequences shown in Fig. 2, A and B.

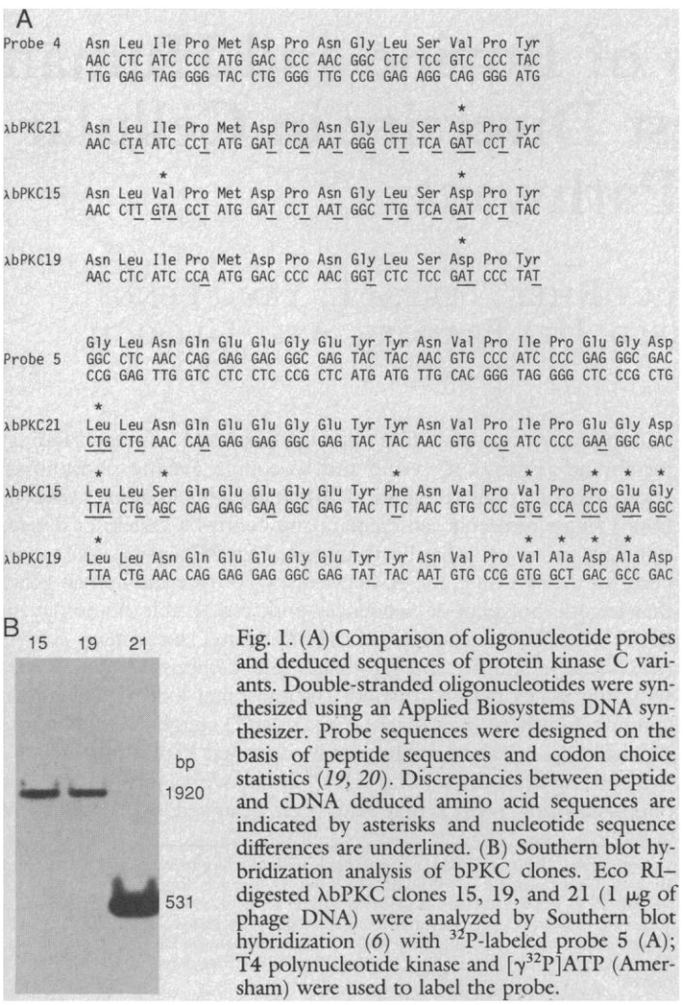

Fig. 1. (A) Comparison of oligonucleotide probes and deduced sequences of protein kinase C variants. Double-stranded oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer. Probe sequences were designed on the basis of peptide sequences and codon choice statistics (*19, 20*). Discrepancies between peptide and cDNA deduced amino acid sequences are indicated by asterisks and nucleotide sequence differences are underlined. (B) Southern blot hybridization analysis of bPKC clones. Eco RI–digested λbPKC clones 15, 19, and 21 (1 μg of phage DNA) were analyzed by Southern blot hybridization (*6*) with ^{32}P-labeled probe 5 (A); T4 polynucleotide kinase and [γ^{32}P]ATP (Amersham) were used to label the probe.

Protein kinase Cβ cDNA (Fig. 2A) contains an open reading frame coding for 673 amino acids starting with an initiation codon (ATG) at position 65, which is flanked by sequences matching Kozak's criteria (*8*) for translation initiation. The encoded polypeptide has a calculated molecular size of 76.9 kD. The coding region is flanked by 64 nucleotides of GC-rich, presumably 5′ noncoding sequence and 880 nucleotides of 3′ untranslated sequence, including a stretch of eight adenylate residues, which may be part of a poly(A) tail. However no AATAAA polyadenylation signal is found at the usual distance (15 to 20 nucleotides) upstream, suggesting that this cDNA was primed at an internal A stretch and that the corresponding mRNA extends further 3′. The presence of a potential polyadenylation signal at position 2623 (AATAAA) suggests the possibility of alternative poly(A) addition and thus generation of multiple mRNA's.

Bovine γ-type protein kinase C is encoded by the 2168-nucleotide cDNA inserts of λbPKCγ551 and λbPKCγ19, which include an open reading frame of 2046 nucleotides and 362 nucleotides of 3′ untranslated sequence (Fig. 2B). The coding portion of our bPKCγ cDNA appears to be incomplete at its 5′ end, with 16 amino acid codons missing if we assume a colinear organization with α and β PKC types. In this case bovine PKCγ would be 697 amino acids long with an approximate molecular size of 78.4 kD.

Structural features of the protein kinase C family. Alignment of bovine PKCα, -β, and -γ sequences reveals an overall sequence homology in two major domains that are separated by one large variable region (V3) (Fig. 3, A and B). Overall, bPKCγ exhibits the highest degree of divergence within five significantly variable regions designated V1 to V5 (Fig. 3, A and B). The V1 region is comprised of 16 amino acid residues that follow a Met-Ala-Asp amino-terminal tripeptide, which is conserved in bPKCα and -β. The region between V1 and V2 (Fig. 3A, C1) is highly conserved in all three types, with 81 percent identity and mostly conservative changes. Here 12 conserved cysteine residues comprise a 50-residue sequence repeat containing six cysteines each, as described by Parker *et al.* (*6*).

The variable region V2 is identified by the presence in bPKCγ of 14 amino acid residues that are entirely unrelated to the bPKCα and -β sequences. Both α and β types contain 9 of 13 differences in this region. The 115-residue conserved region (C2) that follows V2 (bPKCα 175 to 290) is 67 percent identical in all bPKC types; more than 65 percent of the differences represent conservative changes.

Parker *et al.* (*6*) described a potential calcium-binding domain in bPKCα that has a high degree of structural homology with calmodulin (*9*). Interestingly, this site is not conserved in either bPKCβ or bPKCγ. The sequence 292 to 303, EGDEEGNVELRQ (*10*), is located just within V3 in bPKCα and is similar to that found at this position in bPKCβ; however, the resemblance to the calmodulin prototype calcium-binding loop (*9*) is more distant. An even more divergent sequence is present in the corresponding region of bPKCγ. Since it is not yet known whether the three bPKC proteins display identical calcium dependencies, it is difficult to assess the significance of this divergence. However, if this site is used to bind calcium, divergence may represent functional distinctions within the bPKC family.

Lack of conservation also implicates other potential calcium-binding sites, one of which can be discerned immediately upstream from V3. Although this site is less homologous with a classical calmodulin Ca^{2+}-binding loop (*9*), it is conserved in all three bPKC

Table 1. Correlation of protein kinase C sequences with human chromosomes in somatic cell hybrids (human × rodent).

	CKα	CKβ	CKγ	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Concordant	+/+			1	0	0	0	1	1	0	2	0	0	1	0	1	1	1	0	2	1	2	1	1	2	1	0
	−/−			7	7	2	5	7	3	7	6	8	7	5	5	3	1	5	4	9	5	5	5	2	4	3	9
Discordant	+/−			2	3	3	3	2	2	2	1	3	3	2	3	2	2	2	3	0	2	1	2	1	1	2	3
	−/+			2	2	6	3	2	6	2	3	1	2	4	4	5	7	4	5	0	4	4	4	7	5	1	0
Discordant clones				4	5	9	6	4	8	4	4	4	5	6	7	7	9	6	8	0	6	5	6	8	6	3	3
Informative clones*				12	12	11	11	12	12	11	12	12	12	12	12	11	11	12	12	11	12	12	12	11	12	7	12
Percent discordant				33	42	82	55	33	66	36	33	33	42	50	58	64	82	50	66	0	50	42	50	73	50	57	25
Concordant		+/+		4	6	10	8	5	8	3	8	3	3	6	6	8	12	8	10	3	8	8	6	9	9	4	4
		−/−		7	8	5	6	6	4	4	4	7	7	4	6	3	5	4	8	6	5	5	3	4	3	4	7
Discordant		+/−		6	5	1	4	7	3	9	4	9	9	6	6	4	0	6	0	9	4	4	6	2	3	3	8
		−/+		1	1	3	1	2	4	3	4	1	1	3	2	4	2	2	0	2	3	3	5	4	5	2	1
Discordant clones				7	6	4	5	9	7	12	8	10	10	9	8	8	2	8	0	11	7	7	11	6	8	5	9
Informative clones*				18	20	19	19	20	19	19	20	20	20	19	20	19	19	20	18	20	20	20	20	19	20	13	20
Percent discordant				39	30	21	26	45	37	63	40	50	50	47	40	42	11	40	0	55	35	35	55	32	40	38	45
Concordant			+/+	2	3	5	4	3	7	0	4	0	1	4	3	5	7	2	6	1	5	8	4	7	6	0	1
			−/−	7	7	3	6	6	4	3	3	7	6	4	6	2	3	3	5	7	4	8	3	3	4	4	6
Discordant			+/−	5	5	3	4	5	1	8	2	8	7	4	5	2	3	6	2	7	3	0	4	1	2	3	7
			−/+	0	1	4	1	2	3	4	5	1	2	4	2	6	1	5	2	1	4	0	5	5	4	2	2
Discordant clones				5	6	7	5	7	4	12	7	9	9	8	7	8	4	11	4	8	7	0	9	6	6	5	9
Informative clones*				14	16	15	15	16	15	15	14	16	16	16	16	15	14	16	15	16	16	16	16	16	16	9	16
Percent discordant				36	38	47	33	44	27	80	50	56	56	50	44	53	29	69	27	50	44	0	56	38	38	56	56

*Data for chromosomes involved in rearrangements or present at a frequency of 0.1 or less were excluded.

types, and residues 279 to 287 might form a coiled structure that could coordinate calcium through appropriately spaced amino acid side chains. Further analysis will be required to determine the precise location or locations of the Ca^{2+}-binding site or sites.

The longest region of extensive sequence divergence is directly adjacent to this potential calcium-binding site. Comprised of 47, 50, and 59 residues in bPKCα, -β, and -γ, respectively, this hypervariable V3 region has hydrophilic characteristics in all three bPKC sequences (Fig. 3B), and is likely to represent an exposed surface domain since it is sensitive to proteolytic attack (*6*). We have proposed that V3 represents a hinge region between regulatory and catalytic domains (*6*).

A highly conserved region that includes a Gly X Gly XX Gly (where X is any amino acid) consensus sequence for adenosine triphosphate (ATP) binding (*11*) lies immediately beyond the V3 region, and marks the beginning of the kinase domain (Fig. 3A). Extensive sequence homology (about 72 percent) is found in this amino terminal half of all three bPKC's with only a minor insertion of five residues in the bPKCγ sequence (position 411, V4). Interestingly, a second Gly X Gly XX Gly consensus sequence for ATP-binding sites is found in all three bPKC sequences at the end of the protein kinase domain (positions: α, 583; β, 585; γ, 599) with lysine residues found at an appropriate distance (17 amino acids) downstream in bPKCα and -β only. An arginine residue is located in the corresponding position in bPKCγ. Whether or not these sequences represent an additional ATP-binding site requires further investigation. Another minor divergent structural feature is found at the carboxyl terminus (V5): α and γ are 2 and 11 residues longer, respectively, than the aligned bPKCβ sequence.

Analysis of the hydropathic characteristics of PKCα and its relatives (Fig. 3B) further demonstrated the extensive overall similarity of these molecules, but did not provide additional clues with regard to the location of a structural domain involved in plasma membrane interactions. Only the cysteine-rich repeat region contains short hydrophobic stretches that may, as part of an appropriately folded conformation, permit transient association with the cytoplasmic face of the plasma membrane.

Interspecies conservation of the protein kinase C family. Comparison of bPKC family sequences may provide clues regarding the significance of bPKC variable sequences. In related, but functionally distinct polypeptides such as the receptor tyrosine kinases (*12*) divergent sequences are thought to be involved in the definition of specificity for ligand binding and signal generation, yet in proteins such as proinsulin and prorelaxin, variable regions serve as mere spacer sections to facilitate protein folding (*13*). To obtain evolutionary clues to this question and to verify the existence of protein kinase C–related sequences in other species, we isolated human protein kinase C cDNA clones from a human fetal brain λgt10 cDNA library (1.5×10^6 clones). The library was screened at high stringency with type-specific oligonucleotides obtained from V3 regions (legend to Fig. 2, A and B) and revealed the existence of protein kinase C α-, β-, and γ-type sequences in humans. Partial human protein kinase C α, β, and γ clones (λhPKCα7; λhPKCβ15, λhPKCβ802; λhPKCγ6) were isolated and characterized by nucleotide sequence analysis (see Fig. 2, A and B).

Amino acid sequence conservation was found to be more than 98 percent between bovine and human gene products for all three types of PKC within the regions covered by our clones (for α, 98.4 percent, reflecting seven amino acid substitutions between positions 16 and 444, Asp^{36}, Lys^{38}, Gln^{222}, Asn^{224}, Val^{242}, Met^{299}, Lys^{325}; for β, 98.4 percent; for γ, 99.3 percent). Nucleotide sequence conservation was lower in all three cases because of a large number of silent mutations (α, 89 percent; β, 93.5 percent; γ, 92.3 percent). The high degree of sequence conservation of PKCβ between bovine brain and human fetal brain sequences extends beyond the termination codon for 341 nucleotides (89 percent) into the 3′ untranslated region (Fig. 2A), at which point they diverge. The extensive sequence conservation within all regions of bovine and human α, β, and γ PKC gene products strongly supports the idea that the variable domains of different PKC types play an important role in defining distinct functional specificities of these proteins, rather than functioning as mere structural spacer sequences.

Chromosomal assignment of protein kinase C genes. To establish the genetic localization of the PKC gene family members, the chromosomal assignments of human genes for PKCα, PKCβ, and PKCγ were made by in situ hybridization and by Southern analysis of somatic cell hybrids (*7*). In situ hybridization of a ^3H-labeled bPKCα probe (a 504-bp Eco RI insert of λbPKCα21) to

human chromosomes yielded silver grains at bands q22 → q24 of chromosome 17 in 24 out of 110 cells (21.8 percent) (Fig. 4A). Of the 188 total grains observed, 27 (14.4 percent) were found over this specific region (Fig. 4B).

Metaphase cells (105) were analyzed after in situ hybridization with bPKCβ probe (a 1921-bp Eco RI fragment from λbPKCβ15); 30 (28.6 percent) had silver grains over the p12 → q11.1 bands of chromosome 16 (Fig. 4C). Grains at this specific region represented 13.2 percent (31 of 234) of all chromosomal label (Fig. 4D).

In situ chromosomal hybridizations with the bPKCγ probe

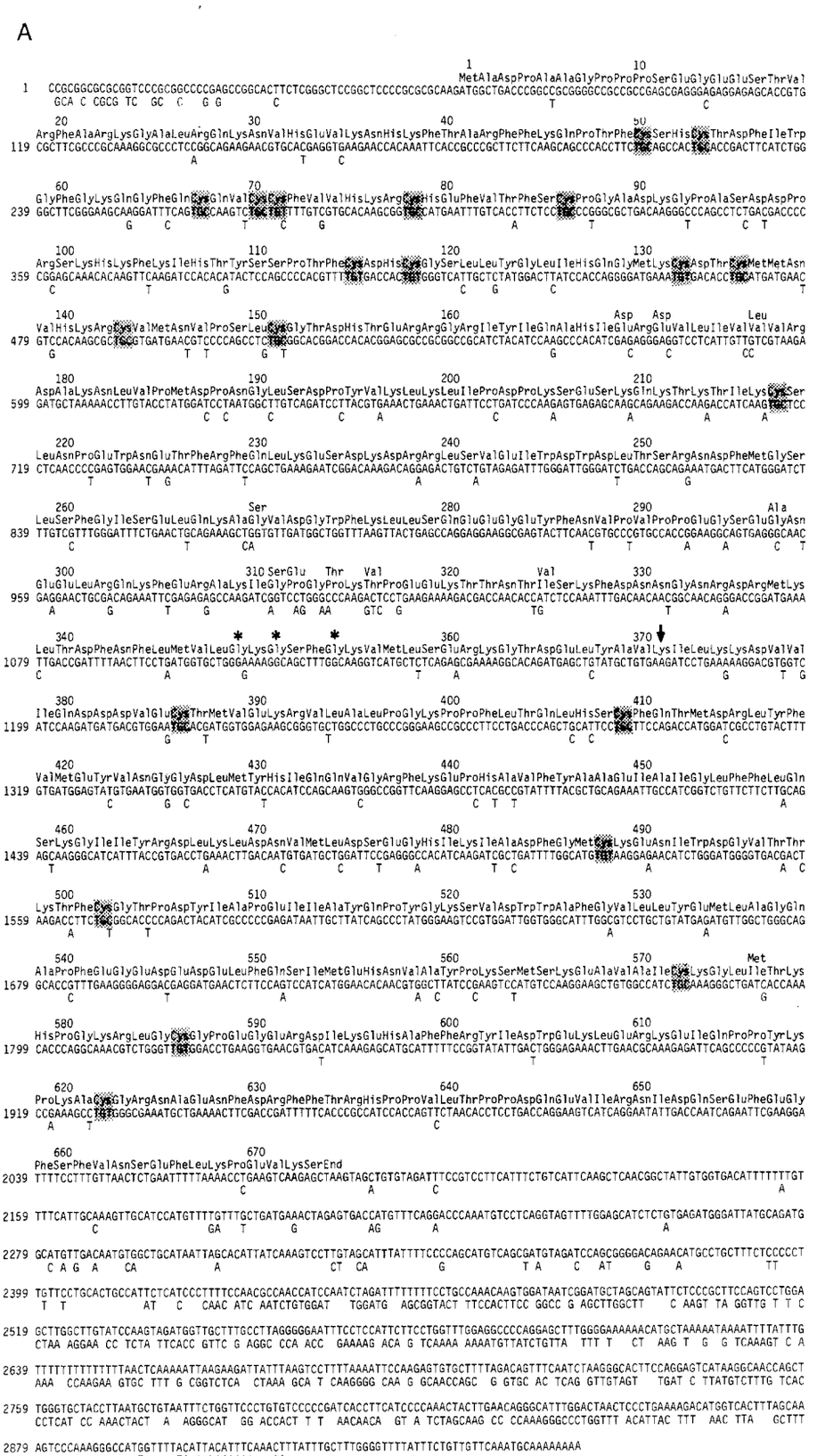

Fig. 2. (A) Sequences of bovine and human β type protein kinase C. Sequences were derived from bovine brain clones λbPKCβ15 and λbPKCβ831; human PKCβ sequences were obtained from fetal human brain cDNA clones λhPKCβ15 and λhPKCβ802. Cysteine residues are shaded and residues involved in ATP binding are emphasized by asterisks (Gly) or an arrow (Lys). Only differences in the human PKCβ sequence are shown. Clones λhPKCβ15 and 802 were isolated from a human fetal brain cDNA library of 1.5×10^6 independent clones using a bPKCβ specific probe from the V3 region (complementary nucleotides to 996 to 1042 in bPKC). (B) Protein kinase C γ-type sequences from bovine brain and human fetal brain. Clones λbPKCγ551 and 19 resulted in a sequence lacking the 5′ initiation site. Human sequences from λhPKCγ6 are aligned and extend further 5′ (in brackets) while being incomplete at the 3′ end (black dots indicate end of cloned cDNA). An inframe stop codon is underlined in the 5′ human untranslated sequence. Cysteine residues (shaded) and ATP-binding site glycines (asterisks) and lysine (arrow) are emphasized. A PKCγ-specific oligonucleotide probe complementary to the V3 region of bPKCγ was used to isolate the partial human PKCγ cDNA clone λhPKCγ6 (complement of nucleotides 1157 to 1204).

(1912-bp Eco RI fragment from λbPKCγ19) revealed that 30.5 percent (32 of 105) of cells examined had silver grains at the distal long arm of chromosome 19, 19q13.2 → q13.4 (Fig. 4E). Of 200 grains on 105 metaphase cells, 36 (18 percent) were over this specific region (Fig. 4F). No secondary sites of hybridization were found for each probe, and therefore no cross-hybridizations were detected among these three genes (Fig. 4, B, D, and F).

Southern blot analysis of somatic cell hybrid DNA confirmed the localizations of PKCα, PKCβ, and PKCγ to human chromosomes 17, 16, and 19, respectively. ^{32}P-labeled bPKCα-specific sequences were hybridized to Eco RI– or Hind III–digested DNA prepared from 12 hybrids (human × rodent). Major 4.7-kbp Eco RI and 4.2-kbp Hind III fragments were detected only in human control DNA and in hybrids containing human chromosome 17 (the 3.8-kbp Eco RI and 7.5-kbp and 3.0-kbp Hind III human fragments being visible after longer exposure). In addition, the 4.7-kbp human specific Eco RI fragment was also detected in a mouse × human hybrid containing only the q21 → qter region of chromosome 17. The regional assignment of the PKCα gene to human chromosome 17q21 → qter made by Southern blot analysis agreed with the localization (17q22 → q24) determined by in situ hybridization.

Southern blot analysis of DNA from 20 somatic cell hybrids (human × Chinese hamster) was performed with the bPKCβ probe. The hybrids containing human chromosome 16 all generated a 7.5-kbp Hind III human PKCβ hybridizing fragment. Furthermore, human PKCβ sequences were also detected in two subclones of a hybrid containing a rearranged chromosome 16 with a deletion of region 16q22 → qter (*14*). This indicates that the human PKCβ gene is not located at 16q22 → qter, also compatible with the chromosomal assignment (16p12 → q11.1) made by in situ hybridization.

DNA extracted from 16 somatic cell lines (human × Chinese hamster) was hybridized with ^{32}P-labeled bPKCγ probe. The 5.5-kbp, 4.6-kbp, and 4.3-kbp human PKCγ Hind III bands and the 7.2-kbp, 4.4-kbp, and 3.4-kbp human PKCγ Eco RI bands were detected only in hybrids containing human chromosome 19. Dis-

B

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   1 CCTTGCGGAGCCGGCGCGCCCGGGGTGCCGCTCCCTGCCTGGCGCGCTCCGCACCTGGAGGTGCCTTGCCCCCTCTCCTGCCCACCTCGGAATTTCCCTGTGGCTCCTTTGATCCTTCGAG

                                                                                                  1                  10
                                                                                                  MetAlaGlyLeuGlyProGlyValGlyAspSerGluGlyGlyPro
 121 TCTCCAGCTCCTCTCCCTTCCACCTGTTTCCCCCAAGAAAGGCAGGATCCTGGTCCCTGCTACGTTTCTGGGGCCATGGCTGGTCTGGGCCCCGGCGTAGGCGATTCAGAGGGGGGACCC

                 20                  30                  40                  50
     ArgProLeuPheCysArgLysGlyAlaLeuArgGlnLysValValHisGluValLysSerHisLysPheThrAlaArgPhePheLysGlnProThrPheCysSerHisCysThrAspPhe
 241 CGGCCCCTGTTCTGCAGAAAGGGGGCTCTGAGGCAGAAGGTGGTCCATGAGGTCAAGAGCCACAAGTTCACCGCTCGCTTCTTCAAGCAGCCGACCTTCTGCAGCCACTGCACTGACTTC
                                                 C  A                                C                   C

                 60                  70                  80                  90
     IleTrpGlyIleGlyLysGlnGlyLeuGlnCysGlnValCysSerPheValValHisArgArgCysHisGluPheValThrPheGluCysProGlyAlaGlyLysGlyProGlnThrAsp
 361 ATATGGGGGATTGGAAAACAGGGTCTGCAATGTCAAGTCTGCAGTTTTGTGGTTCATCGACGATGCCACGAGTTTGTGACCTTCGAGTGTCCAGGCGCTGGGAAGGGCCCCCAGACGGAC
       C    T C    G    C                   C                   A

                100                 110                 120                 130
     AspProArgAsnLysHisLysPheArgLeuHisSerTyrSerSerProThrPheCysAspHisCysGlySerLeuLeuTyrGlyLeuValHisGlnGlyMetLysCysSerCysGluGlu
 481 GATCCCCGGAACAAGCACAAGTTCCGTCTGCACAGCTACAGCAGCCCCACCTTCTGCGACCACTGTGGCTCCCTGCTCTACGGGCTGGTGCACCAGGGCATGAAGTGTTCTTGCGAAG
       C          A          C    T                   C          T          A

                140                 150                 160                 170
     MetAsnValHisArgArgCysValArgSerValProSerLeuCysGlyValAspHisThrGluArgArgGlyArgLeuGlnLeuGluIleArgAlaProThrSerAspGluIleHisVal
 601 ATGAACGTGCACCGGCGCTGTGTGCGCAGCGTGCCCTCTCTGTGCGGCGTGGACCACACGGAGCGCCGGGGCCGCCTGCAGCTGGAGATCCGGGCGCCCACTTCCGATGAGATCCACGTT
                         T                   T          C    C G                T    AG A          A

                180                 190                 200                 210
     ThrValGlyGluAlaArgAsnLeuIleProMetAspProAsnGlyLeuSerAspProTyrValLysLeuLysLeuIleProAspProArgAsnLeuThrLysGlnLysThrArgThrVal
 721 ACGGTTGGCGAGGCCCGGAACCTCATCCCAATGGACCCCAACGGTCTCTCCGATCCCTATGTGAAGCTGAAGCTCATCCCAGACCCTCGGAATTTGACCAAGCAGAAGACCCGCACGGTG
       T              T    A T T          T       T          A                        CC   G A          A

                220                 230                 240                 250
     LysAlaThrLeuAsnProValTrpAsnGluThrPheValPheAsnLeuLysProGlyAspValGluArgArgLeuSerValGluValTrpAspTrpAspArgThrSerArgAsnAspPhe
 841 AAAGCTACGCTAAACCCTGTGTGGAACGAGACCTTTGTGTTCAACCTGAAGCCGGGGGGACGTGGAGCGCCGGCTCAGCGTGGAGGTGTGGGACTGGGACCGGACCTCCCGAAACGACTTC
       C                   T                   A    T                                                  C

                260                 270                 280                 290
     MetGlyAlaMetSerPheGlyValSerGluLeuLeuLysAlaProValAspGlyTrpTyrLysLeuAsnGlnGluGlyGluTyrTyrAsnValProValAlaAspAlaAspAsn
 961 ATGGGCGCCATGTCCTTCGGCGTCTCGGAGCTGCTCAAGGCGCCGGTGGACGGCTGGTACAAGTTACTGAACCAGGAGGAGGGCGAGTATTACAATGTGCCGGTGGCTGACGCCGACAAC
       G          T                   C    T                                                             T T

                300                 310         ValSer  ●●●  320                 330
     CysAsnLeuGluGlnLysPheGluAlaCysAsnTyrProLeuGluLeuTyrGluArgValArgThrGlyProSerSerSerProIleProSerProSerProSerProThrAspSerLys
1081 TGCAACCTCCTCCAGAAGTTCGAGGCCTGTAACTACCCCCTGGAACTATACGAGAGGGTGCGGACGGGTCCCTCTTCATCTCCCATCCCCTCCCCATCCCCCAGTCCCACCGACTCCAAG
       G          T    T                T G  T   GT AGTA A  C●●●

                340                 350                 360                 370
     ArgCysPhePheGlyAlaSerProGlyArgLeuHisIleSerAspPheSerPheLeuMetValLeuGlyLysGlySerPheGlyLysValMetLeuAlaGluArgAlaGlySerAspGlu
1201 CGCTGTTTCTTCGGGGCCAGCCCTGGACGACTGCACATCTCCGACTTCAGCTTCCTCATGGTTCTAGGAAAAGGCAGTTTTGGGAAGGTGATGCTGGCCGAGCGCCGGGGCTCCGATGAG
                                                    *   *   *

                ↓   390                 400                 410
     LeuTyrAlaIleLysIleLeuLysLysAspValIleValGlnAspAspAspValAspCysThrLeuValGluLysArgValLeuAlaLeuGlyGlyArgGlyProGlyGlyArgProHis
1321 CTCTACGCCATCAAGATCCTGAAGAAAGACGTGATCGTCCAGGATGACGACGTGGACTGCACCCTGGTGGAGAAACGCGTGCTGGCTCTGGGGGGCCGAGGCCCGGGAGGCCGGCCGCAC

                420                 430                 440                 450
     PheLeuThrGlnLeuHisSerThrPheGlnThrProAspArgLeuTyrPheValMetGluTyrValThrGlyGlyAspLeuMetTyrHisIleGlnLeuGlyLysPheLysGluPro
1441 TTCCTCACCCAGCTTCACTCCACCTTCCAGACCCCGGATCGCCTGTATTTTGTGATGGAGTATGTCACCGGGGGCGACTTGATGTACCACATTCAACAGCTGGGCAAGTTTAAGGAACCC

                460                 470                 480                 490
     HisAlaAlaPheTyrAlaAlaGluIleAlaIleGlyLeuPhePheLeuHisAsnGlnGlyIleIleTyrArgAspLeuLysLeuAspAsnValMetLeuAspAlaGluGlyHisIleLys
1561 CACGCAGCGTTCTACGCTGCAGAAATCGCCATCGGCCTCTTCTTCCTCCATAACCAGGGCATTATCTATCGGGACCTGAAACTGGACAACGTGATGCTGGATGCCGAAGGACACATCAAA

                500                 510                 520                 530
     IleThrAspPheGlyMetCysLysGluAsnValPheProGlySerThrThrArgThrPheCysGlyThrProAspTyrIleAlaProGluIleIleAlaTyrGlnProTyrGlyLysSer
1681 ATCACCGACTTCGGCATGTGTAAGGAGAACGTCTTTCCCGGGAGTACCACTCGCACCTTCTGCGGGACCCCGGACTACATAGCCCCCGAGATCATTGCCTACCAACCCTATGGGAAGTCT

                540                 550                 560                 570
     ValAspTrpTrpSerPheGlyValLeuLeuTyrGluMetLeuAlaGlyGlnProProPheAspGlyGluAspGluGluLeuPheGlnAlaIleMetGluHisAsnThrValThrTyrPro
1801 GTGGATTGGTGGTCCTTTGGGGTTCTGCTCTACGAGATGTTGGCAGGACAGCCCCCCTTTGATGGAGAAGATGAGGAGGAGCTGTTTCAAGCCATCATGGAACAAACTGTCACCTACCCC

                580                 590                 600                 610
     LysSerLeuSerArgGluAlaValAlaIleCysLysGlyPheLeuThrLysHisProAlaLysArgLeuGlySerGlyProAspGlyGluProThrIleArgAlaHisGlyPhePheArg
1921 AAGTCGCTTTCCCGGGAAGCTGTGGCCATCTGCAAGGGGTTCCTCACCAAGCACCCGGCCAAGCGCCTGGGCTCAGGCCCCGATGGAGAAGCCCACCATCCGCGCTCACGGCTTTTTCCGC

                620                 630                 640                 650
     TrpIleAspTrpAspArgLeuGluArgLeuGluIleAlaProProPheArgProArgProCysGlyArgSerGlyGluAsnPheAspLysPhePheThrArgAlaAlaProAlaLeuThr
2041 TGGATCGACTGGGACAGGCTGGAACGATTAGAGATCGCGCCTCCGTTCAGACCCCGCCCGTGTGGCCGCAGCGGCGAGAACTTCGACAAGTTCTTCACTCGGGCGGCGCCGGCGCTGACA

                660                 670                 680                 690
     ProProAspArgLeuValLeuAlaSerIleAspGlnAlaGluPheGlnGlyPheThrTyrValAsnProAspPheValHisProAspAlaArgSerProIleSerProThrProValPro
2161 CCCCCTGACCGCCTGGTTCTGGCCAGCATCGACCAGGCTGAGTTCCAGGGCTTCACCTATGTCAACCCGGATTTCGTGCACCCGGATGCCCGCAGCCCCATCAGCCCAACGCCTGTGCCA

     ValMetEnd
2281 GTCATGTAATCCCACCTGCCGCCACCAGGCGTCCCCACGGCTCCCTCCTCCGCCCCGGCTTTGGCCCTCGCCTCACCATGCCACCCGCCTTTCCAATTCTAGATATGGCTCCCCAGCGTT

2401 CTGGCCTC
```

cordancy analysis of human chromosomes and hybridization with these three different PKC probes are summarized in Table 1.

Genetic complexity of PKC gene family. Northern (*15*) and Southern blot (*7*) hybridization experiments were carried out to characterize the transcription pattern of the PKC family and to further investigate its genetic complexity. Hybridization analysis of bovine and rat brain total poly(A)-containing RNA's (*16*) resulted in detection of multiple mRNA's by specific bPKCα, -β, and -γ cDNA probes. Surprisingly, despite the close sequence homology between PKC types we observed no cross-hybridization at the high stringency conditions used. Very low levels of mRNA's were found in whole calf brain, while a more intense signal of the same hybridization pattern was obtained with poly(A)-containing RNA from calf cerebellum (Fig. 5A). The hybridization patterns obtained with a bPKCα probe (8.1-kb, 3.8-kb, and 3.0-kb mRNA's) (*6*), compared with those obtained when bPKCβ and bPKCγ probes were used, are shown in Fig. 5A. A 1921-bp Eco RI fragment of λbPKCβ15 detected 8.7-kb, 3.4-kb, and 2.6-kb RNA's in bovine cerebellum, while whole rat brain contained 8.7-kb and 2.6-kb mRNA's with sufficient sequence homology to the β probe to yield a signal under the high stringency conditions used. Use of a synthetic bPKCβ V3 probe (βs) in a Northern hybridization analysis of bovine cerebellum poly(A)$^+$ RNA yielded an identical pattern (Fig. 5A), suggesting that all hybridizing bands contained not only closely related but actual PKCβ sequences, although the possibility of a splicing variant cannot be excluded. A major PKCγ mRNA (1912-bp Eco RI fragment from λbPKCγ19) of 3.1 kb and a minor species of 2.4 kb were found in bovine cerebellum; only the 3.1-kb mRNA was detected in rat brain.

To investigate the possible existence of an even larger number of protein kinase C family genes, we carried out Southern blot analysis of bovine and human genomic DNA with cDNA probes of bovine PKCα, -β, and -γ 3′ untranslated regions (Fig. 5B). One strongly hybridizing band was detected with each bPKC probe in Pvu II–digested DNA from bovine cerebellum (α, 2.3 kbp; β, 8.6 kbp; γ, 10.5 kbp). Furthermore, each bovine DNA lane showed two additional hybridization signals (α, 3.6 kbp and 4.1 kbp; β, 4.7 kbp and 5.8 kbp; γ, 5.7 kbp and 7.6 kbp), which suggests the possible existence of other PKC family genes that are more homologous to α, β, and γ than these three genes are to each other. Weaker bands were detected in Pvu II–digested human liver DNA with bovine PKCα (4.1 kbp and 15.5 kbp), PKCβ (1.3 kbp and 0.8 kbp), and PKCγ (7.2 kbp) cDNA probes. More detailed investigations will be necessary to substantiate the possible existence of additional protein kinase C related genes.

Biological significance of protein kinase C family. We have identified a family of genes that encode polypeptides closely related to protein kinase C. Three members of this gene family have been characterized and were mapped to distinct chromosomal locations. Southern hybridization analysis suggests that an even larger number of PKC genes may exist, and the diversity within this family of proteins may be further increased by alternative splicing in at least some of these genes (*17*). The extensive similarity between the cDNA-deduced primary structures of PKCα, -β, and -γ strongly suggests that the corresponding polypeptides share certain functions including kinase activity. Whether PKCβ and -γ types are also activated by diacylglycerol and calcium remains to be established.

The significance of a family of very similar but distinct PKC molecules bears consideration. Until now, the term protein kinase C has been used to represent what may in fact have been a family of proteins that were indistinguishable by the physical and functional

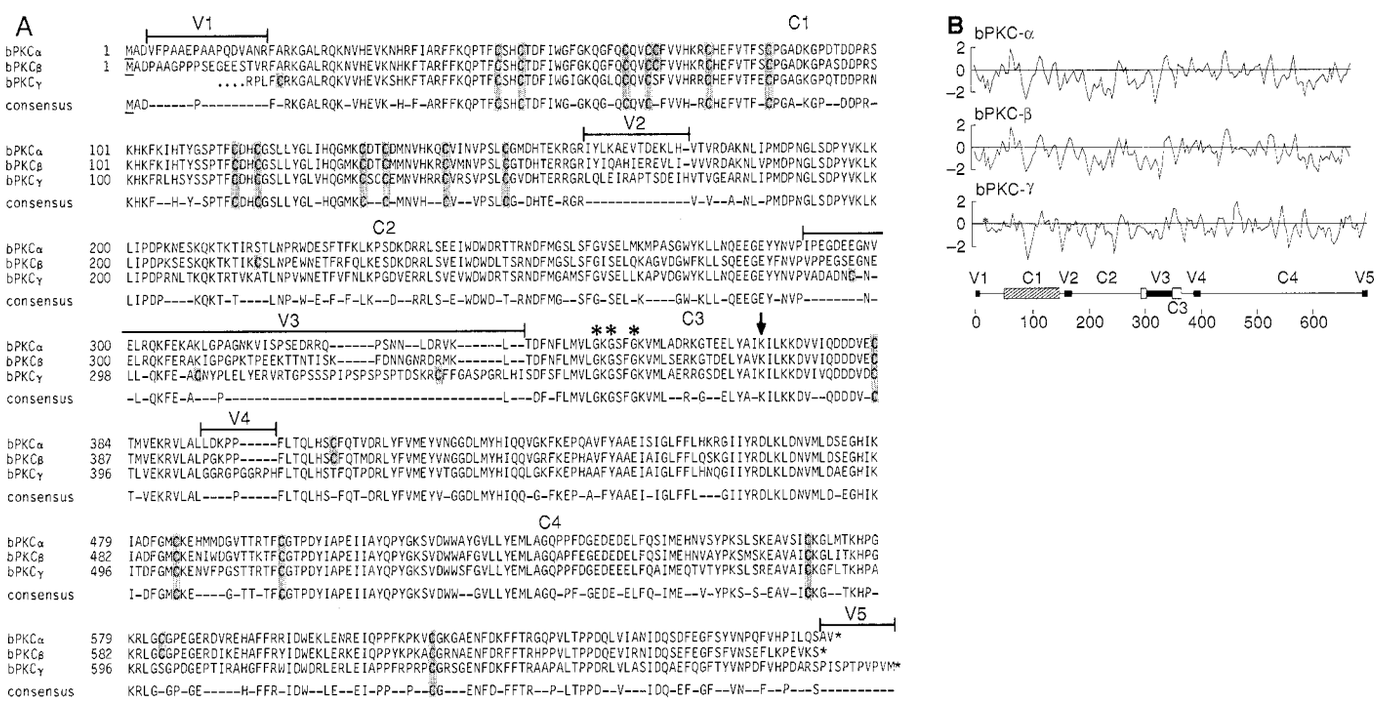

Fig. 3. (A) Comparison of bovine protein kinase C family primary structures. Cysteine residues (shaded) and ATP-binding sites (asterisk and arrow) are emphasized; the consensus line shows identities in PKCα, -β, and -γ, except for the first 16 residues where only α and β are compared. Initiation methionines are underlined and polypeptide chain ends are indicated (asterisk). Variable (brackets) regions V1 to V5 and conserved regions (C1 to C4) are marked. (B) Hydropathy profile comparison of bPKCα, -β, and -γ. Hydrophobicity (above line) and hydrophilicity (below line) values were determined using the computer program of Kyte and Doolittle (*21*). Locations of cysteine-rich repeat domain (hatched box), putative calcium binding site in bPKCα (shaded box), ATP-binding site (open box), variable regions (black boxes) V1 to V3 and conserved regions C1 to C4 are indicated below. The asterisk in the bPKCγ profile indicates incomplete amino-terminal sequences.

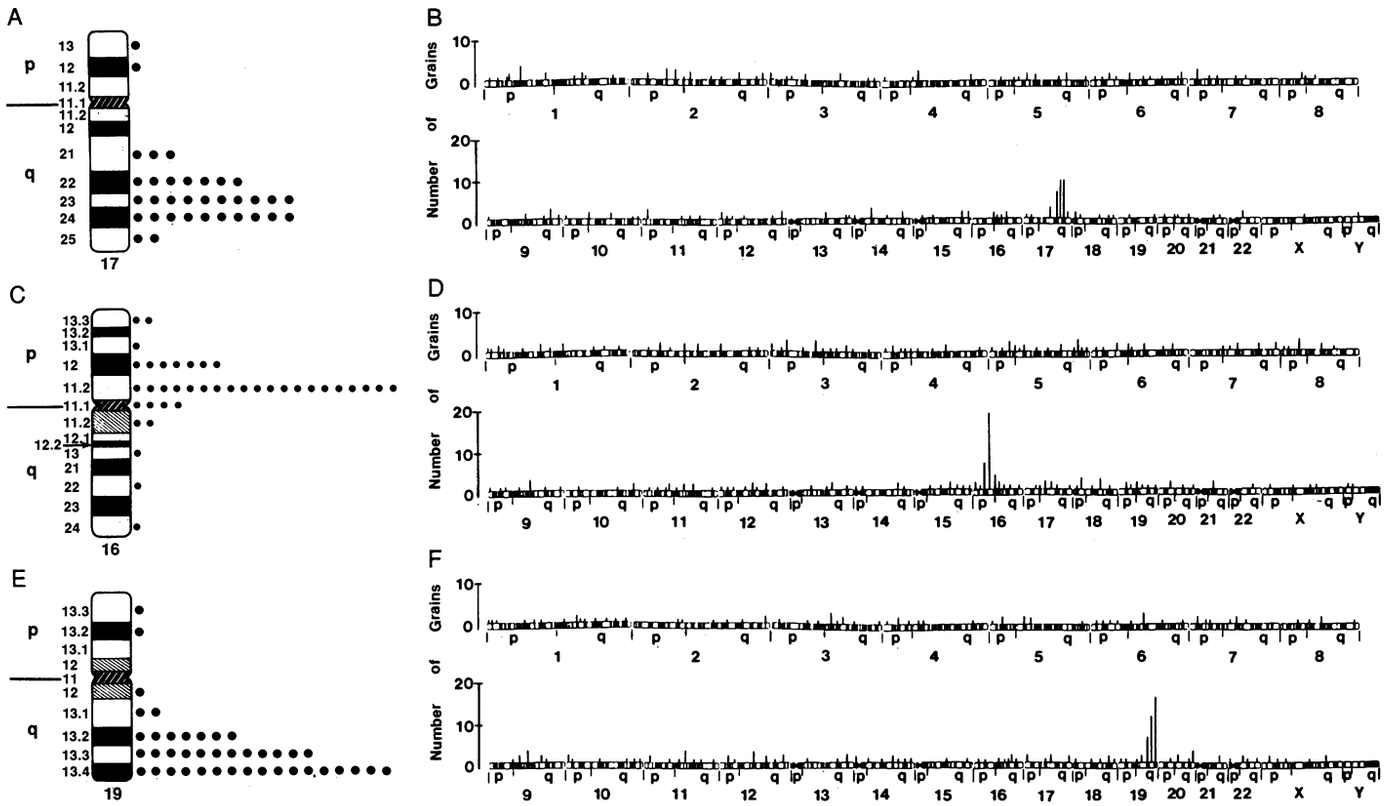

Fig. 4. Silver grain distribution along chromosomes 17, 16, and 19 [ideograms from ISCN 1985 (*22*)] representing specific hybridization to PKCα (A), PKCβ (C), and PKCγ (E). Human metaphase and prometaphase chromosomes were prepared from methotrexate-synchronized peripheral lymphocyte cultures (*23*) of two normal individuals. In situ hybridization was carried out as described (*24, 25*). The probes were ^{3}H-labeled by nick-translation with three labeled nucleotides to specific activities of 2×10^7 to 3×10^7 cpm/g and were hybridized to chromosome preparations at a concentration of 25 ng/ml or 50 ng/ml for 16 hours at 37°C. The emulsion-coated slides were exposed for 10 to 18 days at 4°C. Chromosomes were stained with quinacrine mustard dihydrochloride and photographed under a fluorescence microscope. The chromosomes were then stained for G bands with Wright's stain, and a second photograph was taken of the previously selected cells. The G-banded chromosomes were examined for silver grain localizations. Histograms showing the distribution of silver grains over the human complement for probes bPKCα (B), bPKCβ (D), and bPKCγ (F). Somatic cell hybrids were used to confirm the in situ hybridization results. Their origin and characterization as well as analytical procedures have been reported (*26–31*).

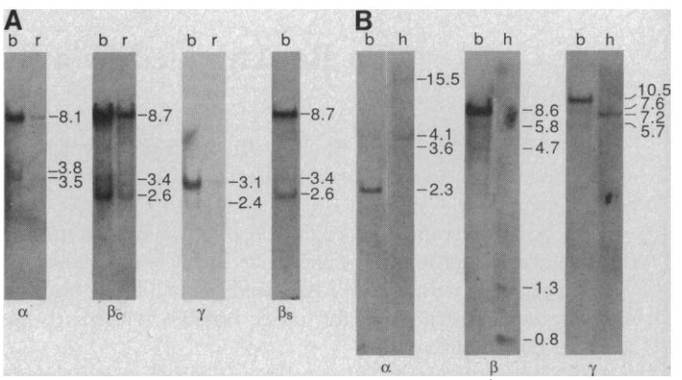

Fig. 5. (A) Northern hybridization analysis of bovine cerebellum (b) and rat whole brain (r) mRNA's. The total poly(A)–containing RNA's (4 μg) (*16*) were separated on agarose-formaldehyde gels and analyzed (*15*). ^{32}P-labeled probes (10^8 cpm/μg) (*32*) from λ clones bPKCα306, bPKCβ15 (1920 bp, β_c) and bPKCγ19 (1912 bp) were used for hybridization. Sizes are given in kilobases and were determined with RNA markers (Bethesda Research Labs). The β_s lane shows a hybridization pattern obtained with a 5′ end-labeled (^{32}P) synthetic oligonucleotide antistrand probe (47 nucleotides) from bPKCβ V3 region (complementary sequence to 996 to 1042). Exposure times were 10 days for α and β_c, 1 day for γ, and 10 days for β_s at −70°C [Cronex Lightning Plus (Dupont)]. (B) Southern blot hybridization analysis of bovine and human chromosomal DNA. Pvu II–digested bovine (b) and human (h) chromosomal DNA (*33*) was analyzed (*6*) with bPKCα, -β, and -γ–specific labeled (*32*) hybridization probes from 3′ terminal cDNA regions (α: 286-bp Hinc II–Eco RI fragment from λbPKCα306; β: 936-bp Eco RI–Eco RI fragment from λbPKCβ15; γ: 170 bp Hinc II–Eco RI fragment from λbPKCγ19) at high stringency conditions (hybridization: 50 percent formamide, 0.75*M* NaCl, 42°C; washing: 0.03*M* NaCl, 45°C) (*33*). Sizes are indicated in kilobase pairs and were determined from λwt size markers (Hind III–Eco RI fragments) (Boehringer-Mannheim).

criteria employed. The existence of a family of protein kinase C molecules suggests that cellular responses may be affected by the activation of one or more polypeptides. This may serve in part to account for the diversity of responses observed and also through differential expression to govern the nature of the response in individual cell types. Preliminary data indicate that individual cell lines do indeed show different patterns of protein kinase C α, β, and γ mRNA and protein expression (*18*).

Distinctive structural features including interspersed variable regions are highly conserved between protein kinase C species, and are thus likely to represent domains that define specific biological functions. They may represent sites of interaction with the specific cell surface proteins, receptors, activators, and substrates that comprise a portion of distinct signaling pathways. The identification and characterization of multiple PKC proteins reported here provide an essential step toward unraveling the mechanisms that regulate lineage-specific cellular responses and specificity of signal transduction within a single cell type.

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