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Expression and Properties of Two Types of Protein Kinase C: Alternative Splicing from a Single Gene

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Two complementary DNA's, encoding the complete sequences of 671 and 673 amino acids for subspecies of rat brain protein kinase C, were expressed in COS 7 cells. The complementary DNA sequence analysis predicted that the two enzymes are derived from different ways of splicing and differ from each other only in the short ranges of their carboxyl-terminal regions. Both enzymes showed typical characteristics of protein kinase C that responded to Ca^{2+}, phospholipid, and diacylglycerol. The enzymes showed practically identical physical and kinetic properties and were indistinguishable from one of the several subspecies of protein kinase C that occurs in rat brain but not in untransfected COS 7 cells. Partial analysis of the genomic structure confirmed that these two subspecies of protein kinase C resulted indeed from alternative splicing of a single gene.

PROTEIN KINASE C (PKC) HAS AN important role in cell surface signal transduction, and it modulates many Ca^{2+}-mediated physiological and pathological processes (*1*). The enzyme was initially thought to be a single entity, but analysis of its complementary DNA (cDNA) clones indicates that PKC is a complex family of closely related structures (*2–7*). Comparison of the predicted amino acid sequences reveals that at least four subspecies of the enzyme may be present in mammalian tissues, particularly in the brain. Although several laboratory groups have isolated the cDNA clones from the libraries prepared from the brains of different mammals including bovine (*2, 3*), human (*3*), rat (*4–6*), and rabbit (*7*), the reported sequences are nearly identical. These cloning experiments have been carried out independently, and thus different, sometimes contradictory, nomenclatures are adopted (Table 1). The three subspecies, α, β, and γ, designated by Coussens *et al.* (*3*) are shown to be encoded on distinct chromosomes, whereas the two subspecies, type I and type II, designated in our earlier report (*4*) are derived from alternative splicing of a single gene, as judged by the analysis of unspliced molecules. Therefore, we use the nomenclatures of α, βI and βII, and γ for the subspecies of PKC as indicated in Table 1.

The βI and βII cDNA's from both rat and rabbit brains encode 671 and 673 amino acid sequences of PKC subspecies, respectively; these species differ from each other only in the carboxyl terminal regions of approximately 50 amino acid residues (*4, 7*). We now report the expression of these cDNA's in COS 7 cells and some of the properties of these enzymes. Huang and his co-workers (*8*) have shown by column chromatography that rat brain PKC may be resolved into apparently three fractions. However, the correspondence of each fraction to the subspecies of PKC predicted by cDNA analyses remains to be explored. The two protein kinases isolated from the COS cells transfected by the βI and βII cDNA's were practically indistinguishable from each other in their physical and kinetic properties, and both appeared to correspond to one of the PKC subspecies present predominantly in brain tissues but not in untransfected COS cells.

The expression plasmids of PKC were constructed as shown in Fig. 1. The cDNA inserts of pTB652 and pTB653 were prepared from the βI and βII cDNA clones,

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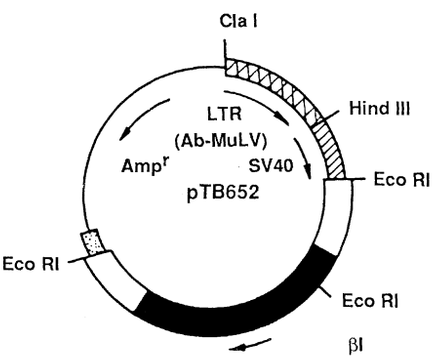


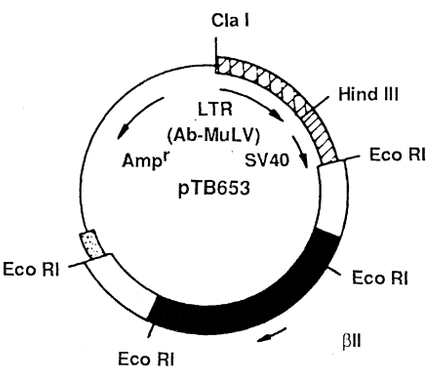


Fig. 1. The structures of two expression plasmids of PKC. The expression plasmid pTB389, containing a single cloning site of Eco RI downstream from the SV40 early promoter and late region introns, was constructed from Okayama and Berg vectors (*14*). The plasmid is essentially similar to pcDL1 (*15*). The Eco RI–Eco RI cDNA fragment (*4*) composed of λCKR152 (nucleotides 1 to 924) and λCKR108 (nucleotides 925 to 3190) of βI cDNA, and that composed of λCKR152 (nucleotides 1 to 924) and λCKR107 (nucleotides 925 to 3406) of βII cDNA were inserted into Eco RI site of pTB389. These cDNA inserts each contained a 5′-noncoding region of 684 nucleotides, the complete coding sequence of the βI or βII cDNA, and a 3′-noncoding region. To enhance promoter activity, we used the Cla I–Hind III fragment [(1.1 kb), which was derived from the original Cla I–Pst I fragment, and the Pst I site was effectively modified by the addition of the Hind III linker] of the Abelson murine leukemia virus (Ab-MuLV) long terminal repeat (LTR) (*16*) and inserted it upstream of the SV40 early promoter region. The resulting expression plasmids are referred to as pTB652 [(**left**) for βI type] and pTB653 [(**right**) for βII type], respectively. The plasmids pTB707 and pTB708 differed from pTB652 and pTB653, respectively, only in the length of the 5′-noncoding region (55 nucleotides). The pBR322 origin of DNA replication and the β-lactamase gene (Amp^r, ampicillin resistant) segments are represented by the solid lines. The LTR's of Ab-MuLV are the crosshatched boxes. The SV40 origin of DNA replication and the SV40 early promoter regions are indicated by hatched boxes. The 5′- and 3′-noncoding regions of PKC cDNA's are indicated by wide open boxes and the coding regions are indicated by wide solid boxes. The stippled boxes contain the polyadenylational signals. Not all the sites for a particular enzyme are shown.

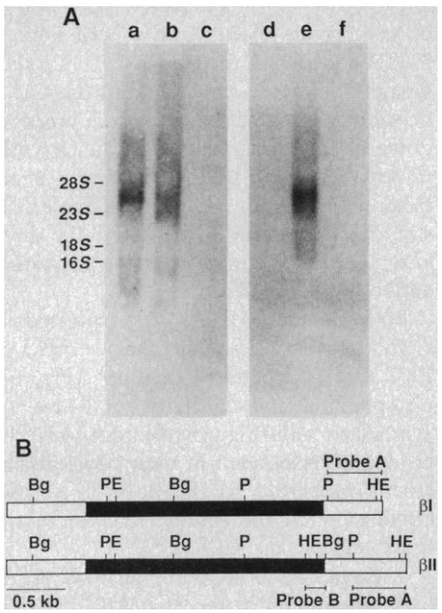


Fig. 2. Northern blot hybridization analysis of transfected COS 7 cells. Fresh monolayers of COS 7 cells, in 10-cm diameter plates (five plates) each containing 10 ml of Dulbecco's modified Eagle's medium containing 5 percent fetal calf serum, were transfected with 30 μg each of the plasmid DNA by the calcium phosphate coprecipitation technique (*17*). After a transfection period of 3.5 hours, the cells were shocked with glycerol for 3 minutes at room temperature (*18*), and fresh medium was added to each plate. After incubation for 18 hours at 37°C, the medium was replaced by 15 ml of fresh Dulbecco's modified Eagle's medium containing 5 percent fetal calf serum. Cells were harvested after additional 48 hours incubation at 37°C; poly(A) RNA's were prepared by guanidine isothiocyanate disruption of the cells with subsequent centrifugation through 5.7*M* cesium chloride (*19*); the RNA was further purified by oligo(dT)-cellulose column chromatography. (**A**) Northern blot analysis. Poly(A) RNA's (5 μg per lane) were fractionated on 1.2 percent formaldehyde-agarose gels, transferred to nitrocellulose filters (*20*), and hybridized to a nick-translated ^{32}P-labeled Pst I–Eco RI 0.45-kb fragment (probe A; lanes a to c) or Hind III–Bgl II 0.16-kb fragment (probe B; lanes d to f). The labeled probes were prepared by nick translation (*21*) of the cDNA fragments. Hybridization was performed for 16 hours at 42°C in 50 percent formamide, 10 percent dextran sulfate, 5× SSPE (1× SSPE is 180 m*M* NaCl, 10 m*M* sodium phosphate, 1 m*M* EDTA at *p*H 7.4), 5× Denhardt's solution, 0.1 percent SDS, and sonicated-denatured salmon sperm DNA (100 μg/ml). The blots were washed in 0.1 percent SDS, 2× SSC (1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate at *p*H 7.0) for 20 minutes at room temperature, and then in 0.1 percent SDS, 0.1× SSC for 30 minutes at 55°C. The dried filters were exposed to x-ray films. (Lanes a and d) pTB652-transfected COS 7 cells; (lanes b and e) pTB653-transfected COS 7 cells; and (lanes c and f) control COS 7 cells. The molecular sizes (*S* units) of the standards are indicated. (**B**) Restriction endonuclease cleavage site maps of the βI and βII cDNA's. Open boxes indicate the 3′- and 5′-noncoding regions. Solid boxes are the coding regions. Horizontal (|—|) show the probe segments used in the blot hybridization experiments. Abbreviations: E, Eco RI; H, Hind III; Bg, Bgl II; and P, Pst I.

Table 1. Isolated cDNA's of PKC. The numbers in parentheses indicate the length (number of amino acids) of deduced amino acid sequences of each clone.

Mammal brain	cDNA's isolated				Reference
Bovine	α (672)		β (673)	γ (697 ?)	(*2, 3*)
Human	α (672)		β (673)	γ (697 ?)	(*3*)
Rat	X* (?)	Type I (671)	Type II (673)		(*4*)
Rat		PKC III (?)	PKC II (673)	PKC I (697)	(*5*)
Rabbit	γ (672)	β (671)	α (673)		(*7*)
Proposed nomenclature:	α	βI	βII	γ	

*Analysis of partial amino acid sequence of a peptide from PKC has predicted the existence of this clone.

respectively, which were isolated from the rat brain cDNA library (*4*). These plasmids each contained a 5′-noncoding region of 684 nucleotides. Plasmids pTB707 and pTB708 were also constructed and are identical to pTB652 and pTB653, respectively, except for the 5′-noncoding region consisting of 55 nucleotides. To detect the messenger RNA's (mRNA's) for these protein kinases in the transfected COS 7 cells, Northern blot hybridization analysis was made (Fig. 2). When probe A, which was common for the βI and βII cDNA's, was used, a positive mRNA was detected in both pTB652- and pTB653-transfected COS 7 cells. In contrast, when probe B, which was specific for the βII cDNA, was used, a corresponding mRNA was detected only in the cells transfected by pTB653. No obviously reactive mRNA was found in the untransfected cells.

High-performance liquid chromatography (HPLC) of the extracts of the pTB652- and pTB653-transfected cells with a Mono Q column revealed a single peak of protein kinase that was dependent on Ca^{2+}, diacylglycerol, and phospholipid (Fig. 3). The activity of this enzyme fraction from the transfected COS 7 cells was approximately three times greater than that from the untransfected (control) cells. If a plasmid having no βI or βII cDNA insert was transfected, increase in enzyme activity was not observed.

Western blot analysis of the fractions (Fig. 4) showed that both pTB652- and pTB653-transfected COS 7 cells contained larger amounts of an 80-kD immunoreactive material that corresponded to authentic PKC which had been purified from the rat brain soluble fraction to a nearly single band (*9*) on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) by DE-52, threonine-Sepharose, and TSK phenyl-5PW column chromatographies (*10*). This authentic preparation was a mixture of multiple subspecies of the enzyme as described below. The above results suggest that both βI and βII cDNA's are transcribed and translated to PKC in the transfected cells. When pTB707

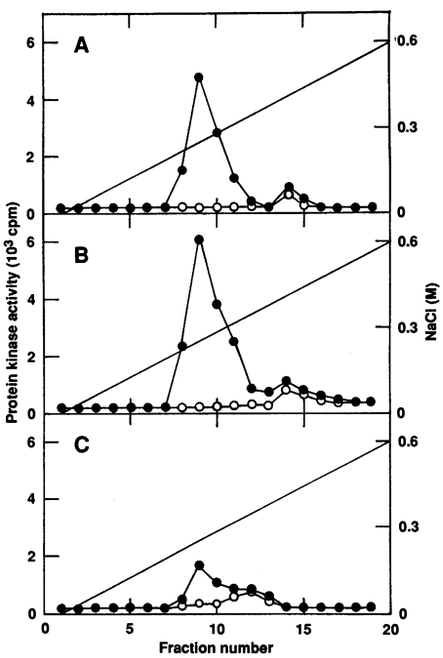


Fig. 3. Mono Q column chromatography of the transfected COS 7 cell extracts. The COS 7 cells (approximately 3×10^7 cells from five plates) were homogenized by sonication (Kontes sonifier) for 1 minute in 1.5 ml of 20 m*M* tris-HCl at *p*H 7.5, containing 0.25*M* sucrose, 10 m*M* EGTA, 2 m*M* EDTA, and leupeptin at 20 μg/ml. The homogenate was centrifuged for 60 minutes at 100,000*g*, and the supernatant (approximately 1.4 mg of protein) was diluted with six volumes of 20 m*M* tris-HCl at *p*H 7.5, containing 0.5 m*M* EGTA, 0.5 m*M* EDTA, and 10 m*M* 2-mercaptoethanol (buffer A), and applied to a Mono Q column (0.5 by 5 cm, Pharmacia HR 5/5) that was connected to a Pharmacia FPLC system and equilibrated with buffer A. Protein kinase C was eluted by application of a 20-ml linear concentration gradient of NaCl (0 to 0.6*M*) in buffer A at a flow rate of 1 ml/min. Fractions (1 ml each) were collected. All procedures above were carried out at 0° to 4°C. The PKC was assayed by measuring the incorporation of $^{32}P_i$ into calf thymus H1 histone from [γ-^{32}P]ATP under the conditions specified earlier (*10, 22*). (**A** to **C**) The pTB652-transfected, pTB653-transfected, and control COS 7 cells, respectively. (●——●) Protein kinase activity in the presence of phosphatidylserine (8 μg/ml), diolein (0.8 μg/ml), and 0.5 m*M* $CaCl_2$; (○——○) Protein kinase activity in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; (——) NaCl.

and pTB708, each containing a short 5′-noncoding region, were transfected to COS 7 cells, essentially the same results were obtained.

The extracts of the pTB707- and pTB708-transfected COS 7 cells were applied to the Mono Q column as described above, and the fractions of the protein kinase were then individually subjected to hydroxyapatite column chromatography (Fig. 5). The enzymes each appeared as two peaks, one major (fractions 28 through 32) and one minor (fractions 43 through 49) (Fig. 5, A and B). The control enzyme preparation from untransfected cells showed only one minor peak (fractions 43 through 49, Fig. 5C). The major peak (fractions 28 through 32) appeared to have originated from the transfected plasmids. But PKC prepared from the soluble fraction of rat brain exhibited three peaks: two major peaks (peak I, fractions 24 through 27; and peak II, fractions 28 through 32) and one minor peak (peak III, fractions 43 through 49) (Fig. 5D). One of the major peaks, peak II, corresponds exactly to the major peak derived from the transfected cells. The three enzyme fractions (peaks I, II, and III) did not appear to represent artifacts during the enzyme purification procedures, and the elution profiles were reproducible. Proteolytic modification can probably be ruled out, since the extraction buffer contained high concentrations of leupeptin as well as both EGTA and EDTA to remove Ca^{2+}. Protein kinase C is highly susceptible to the Ca^{2+}-dependent protease calpain, which breaks it down to small fragments (*1*). The enzymes in peaks I, II, and III each showed a single band with roughly an identical molecular size of 80 kD upon SDS-PAGE. The three enzymes reacted equally with the polyclonal antibody against PKC (*11*).

Both βI and βII enzymes expressed in COS 7 cells showed typical characteristics of PKC that is dependent on Ca^{2+} (Fig. 6), phospholipid, and diacylglycerol. These enzymes were indistinguishable from each other, and corresponded in their physical and kinetic properties to the peak II enzyme obtained from the soluble fraction of rat brain (*12*).

The nucleotide sequence analysis previously suggested that the βI and βII cDNA's of PKC are originated from a single gene by alternative splicing (*4*). To obtain further evidence, analysis of the genomic DNA was made by Southern blot with the Pst I–Eco RI cDNA fragment (Fig. 2, probe A) as a hybridizing probe, which was derived from the 3′-noncoding region. This sequence was chosen because it contained the most rapidly divergent sequence in gene family and

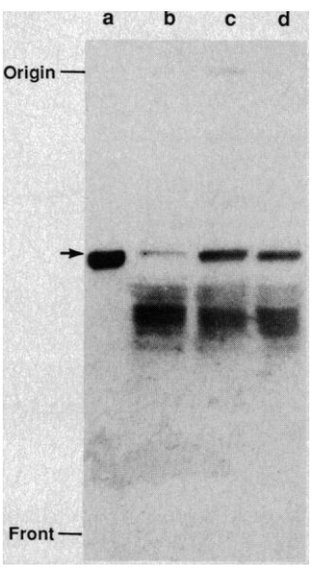


Fig. 4. Western blot analysis of the expression products in COS 7 cells. Portions (30 μg of protein each) of the protein kinase fractions (Fig. 3, fractions 9 to 11) and PKC (0.6 μg of protein)—purified from the soluble fraction of brains from Sprague-Dawley rats by column chromatography on DE-52, threonine-Sepharose, and TSK phenyl-5PW (*10*)—were subjected to 8.5 percent SDS-PAGE (*23*) and transferred to a nitrocellulose filter (*24*). The filter was incubated overnight at 4°C with tris-NaCl buffer (10 m*M* tris-HCl at *p*H 7.5 and 150 m*M* NaCl) containing 5 percent casein and 20 percent normal horse serum and reacted with antiserum to PKC (1:200 diluted in tris-NaCl buffer containing 1 percent casein) for 1 hour at room temperature. The antiserum to PKC was a sample of that described by Parker *et al.* (*2*). The filter was washed with tris-NaCl buffer containing 0.05 percent Tween 20 and incubated with ^{125}I-labeled protein A at 0.1 μCi/ml (9.0 μCi/μg) in tris-NaCl buffer containing 1 percent casein for 1.5 hours at room temperature. The filter was washed as before, dried, and exposed to x-ray film. (Lane a) Rat brain PKC; (lanes b, c, and d) protein kinases from control, pTB652-transfected, and pTB653-transfected cells, respectively. Arrow indicates the position of purified rat brain PKC.

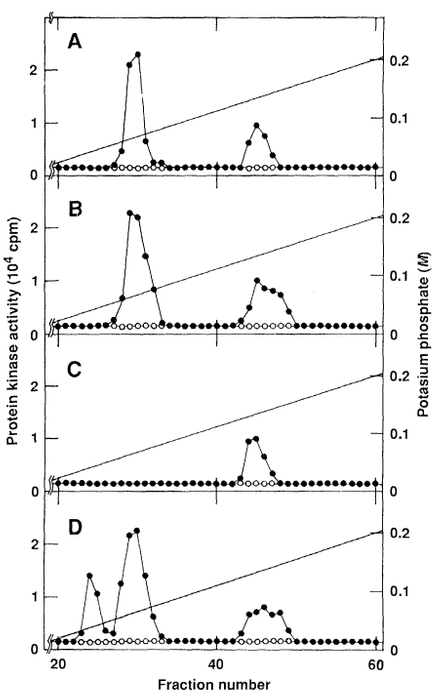


Fig. 5. Hydroxyapatite column chromatography of PKC. The fraction of protein kinase (about 3.5 mg of protein) was prepared from the COS 7 cells (about 5 × 10^8 cells) on a Mono Q column (1 by 10 cm, Pharmacia HR 10/10) as described in Fig. 3, diluted with an equal volume of 20 m*M* potassium phosphate buffer at *p*H 7.5, containing 0.5 m*M* EGTA, 0.5 m*M* EDTA, 10 percent glycerol, and 10 m*M* 2-mercaptoethanol (buffer B), and was applied to a packed hydroxyapatite column (0.78 by 10 cm) (KOKEN, type S, Tokyo), which was connected to a Pharmacia FPLC system and equilibrated with buffer B. Rat brain PKC purified by TSK phenyl-5PW column chromatography (*10*) was then applied to the hydroxyapatite column. The protein kinase was eluted by application of an 84-ml linear concentration gradient of potassium phosphate (20 to 280 m*M*) in buffer B at a flow rate of 0.4 ml/min. Fractions of 1 ml each were collected. The enzyme activity was assayed as described (*10, 22*). (**A** to **C**) Protein kinase from pTB707-transfected, pTB708-transfected, and control COS 7 cells, respectively; (**D**) rat brain PKC. (●——●) Protein kinase activity in the presence of phosphatidylserine at 8 μg/ml, diolein at 0.8 μg/ml, and 0.5 m*M* $CaCl_2$; (○——○) protein kinase activity in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; (——) buffered potassium phosphate.

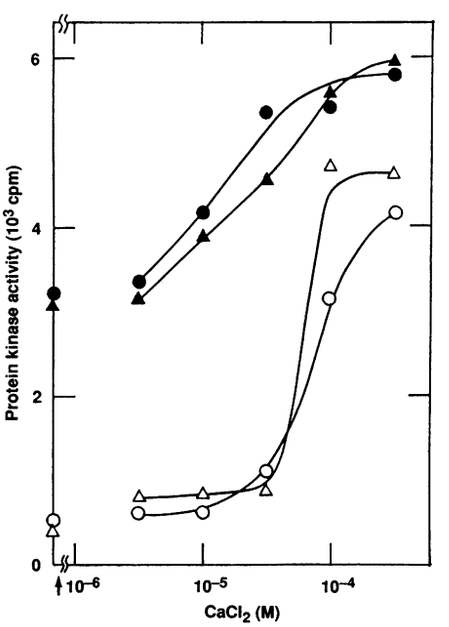
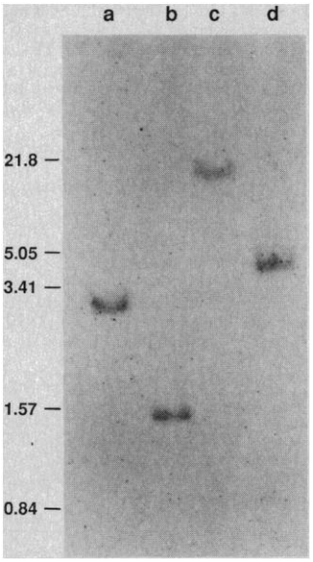


Fig. 6. Effect of Ca^{2+} concentrations on the protein kinases purified partially from the transfected COS 7 cells. The protein kinase was assayed with various concentrations of Ca^{2+} as described (*10, 22*). Where indicated with an arrow, EGTA (1 m*M* at a final concentration) was added instead of $CaCl_2$. (●, ○, ▲ and △) Protein kinase from pTB707- and pTB708-transfected COS 7 cells, respectively. Closed symbols, in the presence of phosphatidylserine and diacylglycerol; open symbols, in the presence of phosphatidylserine alone.

Fig. 7. Genomic Southern blot hybridization analysis. Large molecular size DNA from rat liver was prepared by treatment of the tissue with 0.5*M* EDTA (*p*H 8.0), 0.5 percent Sarkosyl, and proteinase K (100 μg/ml) for 3 hours at 50°C, followed by phenol extraction (*25*). The large molecular size DNA (10 μg) was treated with each restriction endonuclease, fractionated by a 0.8 percent agarose gel, transferred to a nitrocellulose filter, and hybridized to a nick-translated radioactive Pst I–Eco RI 0.45-kb fragment (probe A in Fig. 2B). Conditions of hybridization and washing were same as those for Northern blot hybridization described above. The molecular sizes in kilobase of the standards are indicated. (Lane a) Eco RI; (lane b) Hind III; (lane c) Bgl II; and (lane d) Bam HI.

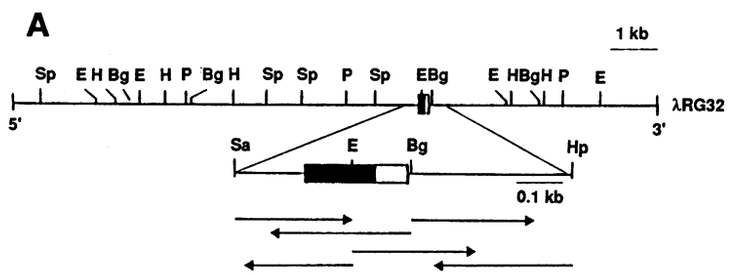


would therefore avoid detection of other closely related genes. This probe always hybridized with a single restriction fragment that was prepared from the rat liver high molecular weight DNA (Fig. 7). The result suggests that the two types of the cDNA's are derived from a single gene.

The βII cDNA is longer than the βI cDNA by means of the insertion of 216 nucleotides as described (*4*). If this insertion occurs by alternative splicing, then the 216-nucleotide sequence must be an independent exon (or exons) in the gene. Therefore, a genomic clone λRG32 (Fig. 8A), which hybridized with the Hind III–Bgl II fragment (Fig. 2, probe B) was isolated, and the nucleotide sequence of the DNA fragment containing this region was determined (Fig. 8B). The inserted sequence was, indeed, an independent exon, and the boundary sequences between the exon and introns agreed with the consensus sequences of splicing donor and acceptor (GT/AG). The results indicate that the βI and βII cDNA's of PKC are in fact derived from the same gene by alternative splicing.

Untransfected COS 7 cells normally contained a relatively high level of the endogenous PKC. The newly expressed βI and βII protein kinases were separable from this endogenous PKC, but eluted together in the fraction indistinguishable from that of peak II of the brain PKC upon chromatography on a hydroxyapatite column (Fig. 5). Although the carboxyl terminal regions of about 50 amino acid residues of βI and βII protein kinases are derived from different mRNA's that are determined by alternative splicing, these regions of the two enzymes still show a strong homology as deduced from the nucleotide sequence analysis of their cDNA clones (*4*). It is practically impossible at present to separate βI and βII protein kinases by conventional enzyme fractionation procedures. Presumably, the peak II PKC that was obtained from rat brain soluble fraction (Fig. 5D) contains both βI and βII protein kinases. During the isolation of the cDNA clones, it was noticed that the frequency of positive clones containing the βII cDNA was about eight times as much as that containing the βI cDNA, implying that in the rat brain the quantity of βII protein kinase is much higher than that of βI protein kinase. Analysis with antibodies specific to each type of enzyme may provide more precise information on the relative ratio as well as the localization of these two species of PKC (*13*).

Our studies show that a specific species of PKC can be expressed in COS 7 cells by introduction of the plasmid containing its defined cDNA molecule. Because the purified enzyme, which was previously thought to be homogeneous, is still a mixture of several subspecies of the enzyme, the studies in which such preparations of a mixture have been used must now therefore be reinterpreted. The enzyme preparation from a transfectant by the defined cDNA described in this report may be useful for further studies of the biological role of a family of PKC in cell surface signal transduction.



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                                                            CysGlyArgAsnAlaGluAsnPheAspArg
                                                                  622
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Fig. 8. Partial DNA sequence analysis of the PKC gene. The rat genomic library (Clontech) was constructed by the insertion of Hae III partially digested rat liver genomic DNA into Eco RI site of Charon 4A vector. The plaque screening was performed as described by Maniatis *et al.* (*26*) and the nick-translated Hind III–Bgl II fragment (Fig. 2, probe B) was used as a probe. Nucleotide sequence analysis was carried out by the chain termination method (*27*) with restriction endonuclease fragments subcloned in the phages M13 vectors. (**A**) Restriction endonuclease cleavage map of the genomic clone λRG32. The intron sequences are indicated by the solid lines. The exon is indicated by boxes (solid box, coding region; open box, 3′-noncoding region). Horizontal arrows show regions and direction of sequencing. Not all the sites for a particular enzyme are shown. Abbreviations: E, Eco RI; H, Hind III; P, Pst I; Bg, Bgl II; Hp, Hpa II; Sa, Sau 3A; and Sp, Sph I. (**B**) Partial nucleotide sequence of the genomic clone λRG32. The exon and intron sequences are represented by capital and lowercase letters, respectively. Numberings of the predicted amino acid residues correspond to those described earlier (*5*).

REFERENCES AND NOTES

1. Y. Nishizuka, *Science* **233**, 305 (1986); U. Kikkawa and Y. Nishizuka, *Annu. Rev. Cell Biol.* **2**, 149 (1986).
2. P. J. Parker *et al.*, *Science* **233**, 853 (1986).
3. L. Coussens *et al.*, *ibid.*, p. 859.
4. Y. Ono *et al.*, *FEBS Lett.* **203**, 111 (1986); Y. Ono *et al.*, *ibid.* **206**, 347 (1986).
5. J. L. Knopf *et al.*, *Cell* **46**, 491 (1986).
6. M. Makowske, M. J. Birnbaum, R. Ballester, O. M. Rosen, *J. Biol. Chem.* **261**, 13389 (1986).

7. S. Ohno *et al.*, *Nature (London)* **325**, 161 (1987).
8. K.-P. Huang, H. Nakabayashi, F. L. Huang, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8535 (1986).
9. The heavy broad band seen in the transfected and untransfected COS cells was not identified. The antibody used was raised against a synthetic peptide with the known sequence commonly present in the subspecies of PKC (*2*).
10. U. Kikkawa, M. Go, J. Koumoto, Y. Nishizuka, *Biochem. Biophys. Res. Commun.* **135**, 636 (1986).
11. Three clones of monoclonal antibody to rat brain PKC were prepared in our laboratories. One of the antibodies reacted with the peak I, but showed a very weak binding activity to peak II or peak III. Other two clones of the antibodies showed different binding activities to these enzyme peaks. Description of the different immunoreactivities of these monoclonal antibodies to subspecies of PKC is in preparation.
12. Knopf *et al.* (*5*) have reported that the protein kinases expressed in the COS cells, PKC I and PKC II, which correspond to γ type and βII type, respectively, required an extremely high concentration of Ca^{2+} (3 m*M*) for their maximal enzymatic activities. Both βI and βII enzymes obtained in our studies were activated by physiologically low concentrations of Ca^{2+} as observed for rat brain PKC.
13. A piece of circumstantial evidence now available suggests that peak I and peak III of PKC shown in Fig. 5 correspond to γ and α protein kinases, respectively (U. Kikkawa *et al.*, in preparation).
14. H. Okayama and P. Berg, *Mol. Cell. Biol.* **3**, 280 (1983).
15. Y. Ebina *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8014 (1985).
16. E. P. Reddy, M. J. Smith, A. Srinivasan, *ibid.* **80**, 3623 (1983).
17. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
18. C. Gorman, R. Padmanabhan, B. H. Howard, *Science* **221**, 551 (1983).
19. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979).
20. H. Lehrach, D. Diamond, J. M. Wozney, H. Boedtker, *ibid.* **16**, 4743 (1977).
21. P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
22. A. Kishimoto, Y. Takai, T. Mori, U. Kikkawa, Y. Nishizuka, *J. Biol. Chem.* **255**, 2273 (1980).
23. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
24. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979).
25. N. Blin and D. W. Stafford, *Nucleic Acids Res.* **3**, 2303 (1976).
26. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), pp. 320–321.
27. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
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Expression of the Multidrug-Resistant Gene in Hepatocarcinogenesis and Regenerating Rat Liver

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Preneoplastic and neoplastic liver nodules and hepatocytes isolated from regenerating rat liver have been shown to be resistant to a broad range of carcinogenic agents. This phenomenon was studied by measuring the expression of the multidrug-resistant (*mdr*) gene in normal liver cells and in preneoplastic and neoplastic nodules and regenerating liver. Levels of messenger RNA for the *mdr* gene, which encodes P-glycoprotein, were elevated in both preneoplastic and neoplastic lesions. Expression of the *mdr* gene also reached high levels in regenerating rat liver 24 to 72 hours after partial hepatectomy. These results show that the expression of the *mdr* gene can be regulated in liver and is likely to be responsible for part of the multidrug-resistance phenotype of carcinogen-initiated hepatocytes and regenerating liver cells.

THE HYPOTHESIS THAT EXPOSURE TO chemical carcinogens results in the development of a population of cells resistant to the cytotoxic effects of carcinogens was first proposed almost 50 years ago (*1*). Farber and his colleagues provided data to support the hypothesis that development of the carcinogen-resistant phenotype is an early step in the neoplastic process, possibly coinciding with initiation (*2*). The model system best studied is the induction of rat hepatomas after treatment with chemical carcinogens (*2, 3*). Normal rat hepatocytes in monolayer culture are highly sensitive to the cytotoxic effects of methotrexate, adriamycin, cycloheximide, and aflatoxin B_1, whereas liver cells isolated from carcinogen-treated rats are resistant to the toxic effects of these agents (*3*). Although the mechanisms of this pleiotropic resistance are not known, some data suggest that the intracellular concentration of both the carcinogen and its metabolites is lower in the resistant cell than in the normal hepatocyte (*4*).

One mechanism that is known to lower intracellular drug concentrations is the expression of the *mdr* gene (*5*), which results in multidrug resistance to hydrophobic natural products such as adriamycin, colchicine, the *Vinca* alkaloids, and actinomycin D (*6*). Full-length complementary DNAs for the human and mouse *mdr* genes have been cloned and used to demonstrate that expression of the *mdr* gene, which encodes a 170,000-dalton membrane glycoprotein (P-glycoprotein), is responsible for multidrug resistance (*7*). Sequence analysis indicates that P-glycoprotein has 12 hydrophobic membrane-spanning regions and two nucleotide-binding domains (*8*). Taken together with direct biochemical evidence that P-glycoprotein binds drugs (*9*), these results are consistent with its function as an energy-dependent drug efflux pump.

Because the P-glycoprotein efflux pump can lower intracellular drug concentrations and because the *mdr* gene is known to be expressed at somewhat increased levels in normal human and rodent liver (*10*), we investigated the possibility that a further increase in expression of this gene might be associated with the development of carcinogen resistance during chemical carcinogenesis and in regenerating rat liver. Our results show increased expression of the *mdr* gene in preneoplastic and neoplastic carcinogen-induced nodules compared to expression in normal rat liver and a dramatic increase in *mdr* messenger RNA (mRNA) levels in regenerating rat liver. These data suggest that expression of the *mdr* gene is regulated in liver and represents a component of the hepatic response to some toxic insults.

As has been shown in normal human liver (*10*), *mdr* RNA is readily detectable in normal rat liver (Fig. 1A, lane 7). The expression of the *mdr* gene was increased in both preneoplastic and neoplastic liver lesions produced by the Solt-Farber method (Fig. 1A, lanes 1 to 6). No apparent difference in *mdr* expression was observed between the early preneoplastic lesions (nodules isolated 6 to 8 weeks after initiation, Fig. 1, A and B, lanes 5 and 6) and neoplastic lesions (nodules isolated 6 to 8 months after initiation; Fig. 1A, lanes 1 to 4). Both fetal and adult rat liver showed lower levels of *mdr* RNA (Fig. 1A, lane 7; Fig. 1B, lanes 1 to 4). The Solt-Farber protocol involves only a single initiating dose of a carcinogen (in our case, diethylnitrosamine, 200 mg/kg, intraperitoneally) followed by a short period of exposure to a low dose of another carcinogen [2-acetylaminofluorene (AAF)] during which

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