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Identification of Members of the Protein Phosphatase 1 Gene Family in the Rat and Enhanced Expression of Protein Phosphatase 1α Gene in Rat Hepatocellular Carcinomas

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We isolated four kinds of cDNA clones of isotypes of catalytic subunits of protein phosphatase 1 (PP-1) from rat liver and testis cDNA libraries. For the cloning, cDNA fragments of *dis2*ml and *dis2*m2, which encode mouse PP-1 catalytic subunits, were used as probes. Two of the four isotypes were thought to be derived from the same gene and produced by alternative splicing. Based on the comparative study of their nucleotide and deduced amino acid sequences with those reported, these cDNA clones were named rat *PP-1α*, *PP-1γ1*, *PP-1γ2* and *PP-1δ*. The deduced amino acid sequences of these four cDNA clones showed about 90% identity. Their amino-terminal regions were highly conserved, and their differences were mainly in the carboxy-terminal regions. Furthermore, several amino acids located in the middle regions of the peptides were conserved in all the isotypes of the catalytic subunits of PP-1, PP-2A, PP-2B and PP-2C. These conserved regions are suggested to be the functional domains of the catalytic subunits of protein phosphatases. Rat hepatocellular carcinomas induced by a food mutagen, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline showed increased expression of *PP-1α*, but no increased expression of *PP-1γ1*, *PP-1γ2* or *PP-1δ*. Involvement of *PP-1α* in hepatocarcinogenesis or in hepatic cell proliferation was suspected.

Key words: Protein phosphatase 1 — Gene family — Alternative splicing — Conserved amino acid

Two groups of serine/threonine-specific protein phosphatase (PP) have been distinguished, protein phosphatases 1 and 2 (PP-1 and PP-2), depending on sensitivity to heat-stable protein inhibitor-1 and inhibitor-2.[1-3] Furthermore, the PP-2 group is distinguishable into three subgroups, PP-2A, PP-2B (calcineurin) and PP-2C, on the basis of differences in dependence on divalent cations.[1-3] All these PPs except PP-2C are composed of catalytic and regulatory subunits,[3] and their substrate specificities are suggested to depend on their subunit conformations.[3-5] The catalytic subunits of PP-1, PP-2A and PP-2B have all been found to have isotypes. For example, *PP-1α* and *PP-1β* in the rabbit,[6-8] *dis2*m1 and *dis2*m2 which encode PP-1 catalytic subunits in the mouse,[9] *PP-2Aα* and *PP-2Aβ* in several species,[10-13] and *PP-2Bα (calcineurin Aα)* and *PP-2Bβ* (*calcineurin Aβ*) in the rat[14,15] have been demonstrated by cDNA cloning. In the case of PP-2C, two isotypes, $PP\text{-}2C_1$ and $PP\text{-}2C_2$, in the rabbit have been identified by isolation of the proteins.[16,17] There are also reports of isolation by low-stringency hybridization of cDNA clones of novel PP catalytic subunits, named *PP-V*,[18] *PP-X*,[19] *PP-Y*[20] and *PP-Z*.[18] The products of *PP-V*, *PP-X*, *PP-Y* and *PP-Z* have low homology to known PP-2A and PP-1 catalytic subunits. However, the reason for the existence of isotypes of each group of catalytic subunits is so far not understood.

To analyze the relevance of PP-2A in carcinogenesis, we have cloned cDNAs for catalytic subunits of PP-2A, *PP-2Aα* and *PP-2Aβ*. We demonstrated the presence of high levels of these mRNAs in rat hepatic tumors induced by a food carcinogen, 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ).[12,13] We also found that NIH3T3 transformants were flattened morphologically by 10 n*M* okadaic acid, a specific and potent inhibitor of PP-2A.[21] Recently, the enzyme activity of phosphatase N, a member of the PP-1 family, was shown to be elevated in rat hepatomas.[22] These results suggest that not only PP-2A but also PP-1 may be involved in carcinogenesis. However, no cDNA clones of the rat PP-1 catalytic subunit have so far been isolated.

In this study, we isolated four kinds of cDNA clones from rat liver and testis cDNA libraries, using *dis2*ml and *dis2*m2 as probes, and compared the nucleotide and amino acid sequences of these cDNA clones with those of cDNA clones derived from other species. We identified a region in which some amino acids are conserved not only

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in isotypes of PP-1 catalytic subunits but also in catalytic subunits of PP-2. Furthermore, we examined expression levels of the four isotypes of PP-1 catalytic subunit mRNAs in rat hepatocellular carcinomas induced by a food carcinogen, 2-amino-3,8-dimethylimidazo[4, 5-*f*]-quinoxaline (MeIQx).

MATERIALS AND METHODS

Isolation of cDNA clones Rat (F344, male, 12 weeks) liver and testis cDNA libraries, constructed in λgt10 (Stratagene)[23, 24] were screened with 1.3 kb and 1 kb *Eco*RI fragments of the coding regions of *dis2*ml and *dis2*m2 cDNAs,[9] respectively. Plaque hybridization was carried out at 42°C in a solution of 50% formamide, 0.65 *M* NaCl, 0.1 *M* sodium PIPES (pH 6.8), 5× Denhardt's solution [1×=0.02% each of Ficoll (Pharmacia), polyvinylpyrrolidone, bovine serum albumin], 0.1% sodium dodecyl sulfate, 5 m*M* EDTA, 10% dextran sulfate, salmon sperm DNA (100 μg/ml) and a probe labeled by the random priming method[25] using [α-^{32}P]dCTP and a Multiprime DNA Labelling System (Amersham), followed by four washes with 2× SSC (1×=0.15 *M* NaCl, 15 m*M* sodium citrate) containing 0.1% sodium dodecyl sulfate at 50°C for 20 min each time. Positive clones were purified and cloned into the *Eco*RI site of Bluescript pKS-M13$^+$ (Stratagene).

Sequence analysis of cDNA clones Both DNA strands were sequenced by the dideoxy chain-termination method using a 7-deaza-Sequenase II kit (United States Biochemical), [α-^{32}P]dCTP and synthetic oligonucleotide primers. The oligonucleotides were synthesized by the phosphoramidite method (Applied Biosystems, model 380A). The strategy used to sequence the cDNA clones is shown in Fig. 1.

Differential hybridization using oligomers as probes Total RNAs from tissues were extracted by a single-step total RNA isolation method.[26] Samples of 10 μg of RNAs were fractionated in formaldehyde/agarose gel and transferred to a nitrocellulose membrane (Schleicher and Schuell) as described.[27] Two kinds of 40-mer oligonucleotides corresponding to the 3′ non-coding region of the cDNA clones shown in Figs. 2b and 2c were synthesized, end-labeled with [γ-^{32}P]ATP with T_4 polynucleotide kinase, and used as probes. Hybridization was performed under the same conditions as plaque hybridization but without dextran sulfate.

Northern blot hybridization using cDNA fragment as probes Samples of 10 μg of total RNAs were blotted and hybridized with the ^{32}P-labeled *Eco*RI-*Eco*RI fragment of *PP-1α*, *Eco*RV-*Pst*I fragment of *PP-1γ*, *Eco*RI-*Eco*RI fragment of *PP-1δ*, and the *Pst*I-*Eco*RI fragment of 3′ non-coding region of the rat *PP-2Aα* cDNA.[12]

MeIQx-induced liver tumors Six-week-old male Fischer 344 rats were obtained from Charles River Japan Inc., Kanagawa. The animals were given a diet containing 0.04% MeIQx. After 40 weeks they were autopsied and tumors were examined for histology. Both tumors obtained from No. 1 and No. 2 rats were well differentiated hepatocellular carcinomas.

RESULTS

Isolation of four kinds of cDNA clones of mRNA for catalytic subunits of PP-1 With a cDNA fragment of *dis2*ml as a probe, four cDNA clones that gave a strong signal were obtained from a rat liver cDNA library composed of 5×10^5 independent clones. All these clones gave the same restriction maps and the clone with the longest sequence of 2.2 kb was used as clone 1 for further analysis. The restriction sites of *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Rsa*I and *Sma*I of clone 1 are shown in Fig. 1.

Three cDNA clones that gave a strong hybridization signal with the *dis2*m2 probe were obtained from the same rat liver cDNA library. These three clones gave the same restriction maps, and the clone with the longest sequence of 2.7 kb was used as clone 2 for further analysis. The map of this clone is shown in Fig. 1.

Five other cDNA clones that hybridized weakly with cDNA fragments of both *dis2*ml and *dis2*m2 were also

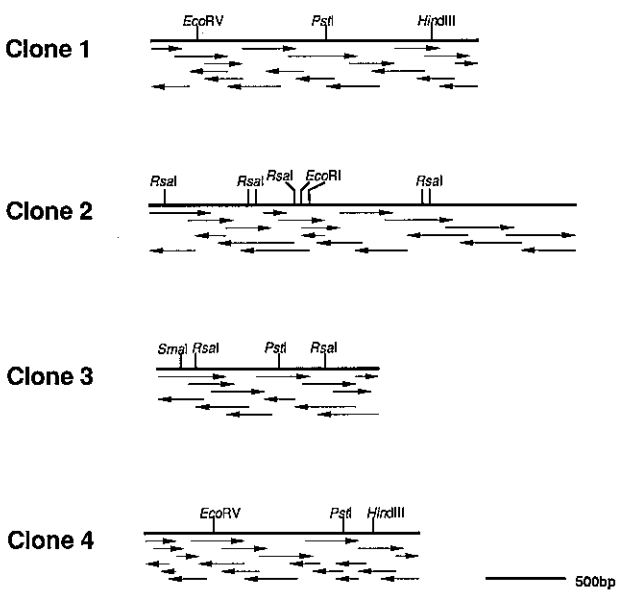

Fig. 1. Restriction maps and strategy used to sequence the cDNA clones of PP-1 catalytic subunits. The arrows show the direction and length of the DNA sequences obtained. Sequences were initiated with Bluescript primers and synthetic oligonucleotide primers.

(a)

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                                                                                CCCGGGAGGCAGGAGAGGGCCCGGAGCTGGTGGGCCGGAGCGGCGGCGCCGC   52

ATGTCCGACAGCGAGAAGCTCAACCTGGATTCCATCATCGGGCGCCTGCTGGAAGTGCAGGGCTCACGGCCTGGAAAGAATGTGCAGCTGACAGAGAACGAGATCCGTGGTCTTTGCCTC  172
M  S  D  S  E  K  L  N  L  D  S  I  I  G  R  L  L  E  V  Q  G  S  R  P  G  K  N  V  Q  L  T  E  N  E  I  R  G  L  C  L    40

AAATCCCGGGAGATTTTCCTGAGCCAGCCTATTCTTCTGGAGCTTGAGGCGCCTCTCAAGATCTGTGGTGACATCCATGGCCAGTACTATGACCTTCTACGGCTGTTCGAGTATGGTGGC  292
K  S  R  E  I  F  L  S  Q  P  I  L  L  E  L  E  A  P  L  K  I  C  G  D  I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y  G  G     80

TTCCCTCCAGAGAGCAACTACCTCTTCTTGGGGGACTATGTGGATCGGGGAAAGCAGTCTTTGGAGACCATCTGCTTGTTGCTGGCCTATAAGATCAAATACCCGGAGAATTTCTTTCTA  412
F  P  P  E  S  N  Y  L  F  L  G  D  Y  V  D  R  G  K  Q  S  L  E  T  I  C  L  L  L  A  Y  K  I  K  Y  P  E  N  F  F  L    120

CTACGTGGGAACCATGAGTGTGCCAGCATCAACCGCATCTATGGCTTCTATGATGAATGCAAGAGACGATACAACATCAAACTGTGGAAGACTTTCACCGACTGCTTCAACTGCCTGCCC  532
L  R  G  N  H  E  C  A  S  I  N  R  I  Y  G  F  Y  D  E  C  K  R  R  Y  N  I  K  L  W  K  T  F  T  D  C  F  N  C  L  P    160

ATTGCAGCCATTGTAGATGAGAAGATCTTCTGCTGCCACGGAGGCCTGTCTCCAGACTTGCAATCCATGGAGCAGATTAGACGTATTATGCGGCCCACGGACGTGCCTGACCAGGGCCTG  652
I  A  A  I  V  D  E  K  I  F  C  C  H  G  G  L  S  P  D  L  Q  S  M  E  Q  I  R  R  I  M  R  P  T  D  V  P  D  Q  G  L    200

TTGTGTGATCTGCTGTGGTCTGACCCTGACAAGGATGTTCAAGGCTGGGGCGAGAATGACCGTGGAGTCTCCTTTACCTTTGGGGCCGAGGTTGTAGCCAAGTTCCTGCACAAGCATGAT  772
L  C  D  L  L  W  S  D  P  D  K  D  V  Q  G  W  G  E  N  D  R  G  V  S  F  T  F  G  A  E  V  V  A  K  F  L  H  K  H  D    240

TTGGACCTCATCTGCAGAGCACATCAGGTTGTAGAAGATGGCTATGAGTTCTTTGCCAAGAGGCAGCTGGTGACACTCTTCTCAGCTCCCAACTACTGTGGCGAGTTTGACAACGCTGGC  892
L  D  L  I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F  A  K  R  Q  L  V  T  L  F  S  A  P  N  Y  C  G  E  F  D  N  A  G    280

GCCATGATGAGTGTGGACGAGACACTCATGTGTTCCTTCCAGATCCTCAAGCCCGCTGATAAGAATAAGGGGAAGTATGGGCAGTTCAGTGGCCTGAACCCCGGAGGCCGTCCCATCACT 1012
A  M  M  S  V  D  E  T  L  M  C  S  F  Q  I  L  K  P  A  D  K  N  K  G  K  Y  G  Q  F  S  G  L  N  P  G  G  R  P  I  T    320

CCACCCCGCAATTCTGCCAAAGCCAAGAAATAGCCTCCATGTGCTGCCCTCTGCCCCAGATGACGGATTATTTGTACAGAAATCATGCTGCCATGGGTCACACTGGCCTCTCAGGCCCAC 1132
P  P  R  N  S  A  K  A  K  K  END                                                                                            330

CCATCATGGGGAACACAGCGTTAAGTGTCTTTCCTTTATTTTTTAAAGAATCAATAGCAGCATCTAATTCCCCAGGGCTCCCTCCCACCAGCACCTGTGGTGGCTGCAAGTGGAATCCTG 1252

GGGCCAAGGCTGCAGCTCAGGGCAATGGCAGACCAGATTGTGGGTCTCCAGCCTTGCATGGCTGGCAGCCAGATCCTGGGGCAACCCATCTGGTCTCTTGAATAAAGGTCAAAGCTGGAT 1372

TCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                                                        1407
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(b)

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                                                                GGGGCTCGTGGCGTGAAGAAGGAGGACGAGTGAGACCCGGGCGCGACGGGGCGCGTGCGGGAGGGTCGGCGGCGGGACGGCG   82

ATGGCGGATATCGATAAACTCAACATCGACAGTATCATCCAACGGCTGCTGGAAGTGAGAGGGTCCAAGCCAGGCAAGAATGTCCAGCTCCAGGAGAATGAAATCCGGGGACTGTGCTTG  202
M  A  D  I  D  K  L  N  I  D  S  I  I  Q  R  L  L  E  V  R  G  S  K  P  G  K  N  V  Q  L  Q  E  N  E  I  R  G  L  C  L    40

AAGTCTCGGGAGATCTTCCTCAGTCAGCCTATCCTTTTAGAACTTGAAGCACCACTCAAGATATGTGGTGACATCCACGGGCAGTACTATGATTTGCTCCGTCTGTTTGAATACGGTGGC  322
K  S  R  E  I  F  L  S  Q  P  E  L  L  E  L  E  A  P  L  K  I  C  G  D  I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y  G  G     80

TTTCCTCCAGAAAGCAACTATTTGTTTCTCGGGGACTATGTGGACAGGGGCAAACAGTCACTAGAGACGATCTGCCTCTTGCTGGCCTACAAAATCAAGTATCCGGAGAACTTTTTTCTT  442
F  P  P  E  S  N  Y  L  F  L  G  D  Y  V  D  R  V  K  Q  S  L  E  T  I  C  L  L  L  A  Y  K  I  K  Y  P  E  N  F  F  L    120

CTTAGAGGGAACCATGAGTGTGCCAGCATCAATAGAATCTACGGATTTTATGATGAGTGTAAGAGAAGATACAACATTAAGCTGTGGAAAACGTTCACAGACTGTTTTAACTGCTTACCG  562
L  R  G  N  H  E  C  A  S  I  N  R  I  Y  G  F  Y  D  E  C  K  R  R  Y  N  I  K  L  W  K  T  F  T  D  C  F  N  C  L  P    160

ATAGCAGCCATCGTGGACGAGAAGATATTCTGCTGTCATGGAGGTTTATCACCAGATCTTCAATCTATGGAGCAGATTCGGCGAATTATGAGACCAACTGATGTACCAGATCAAGGTCTT  682
I  A  A  I  V  D  E  K  I  F  C  C  H  G  G  L  S  P  D  L  Q  S  M  E  Q  I  R  R  I  M  R  P  T  D  V  P  D  Q  G  L    200

CTTTGTGATCTTTTGTGGTCTGACCCCGATAAAGATGTCTTAGGCTGGGGTGAAAATGACAGAGGAGTGTCCTTCACATTTGGTGCAGAAGTGGTTGCAAAATTTCTCCATAAGCATGAT  802
L  C  D  L  L  W  S  D  P  D  K  D  V  L  G  W  G  E  N  D  R  G  V  S  F  T  F  G  A  E  V  V  A  K  F  L  H  K  H  D    240

TTGGATCTTATATGTAGAGCCCATCAGGTGGTTGAAGATGGATATGAGTTTTTTGCAAAGAGGCAGTTAGTCACTCTGTTTTCTGCACCCAACTACTGTGGCGAGTTTGACAATGCGGGC  922
L  D  L  I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F  A  K  R  Q  L  V  T  L  F  S  A  P  N  Y  C  G  E  F  D  N  A  G    280

GCCATGATGAGTGTGGATGAGACCCTCATGTGCTCCTTCCAGATTTTAAAGCCTGCAGAGAAAAAGAAGCCCAATGCCACGAGACCTGTCACACCCCCACGGGGTATGATCACAAAGCAA 1042
A  M  M  S  V  D  E  T  L  M  C  S  G  Q  I  L  K  P  A  E  K  K  K  P  N  A  T  R  P  V  T  P  P  R  G  M  I  T  K  Q    320

GCAAAGAAATAGATGTCACTTGACACTGCCTAGTGGGACTTGTAACATAGCGTACATAACCTTCCTTTTTAAACTGTATGTGTGCTGGTCAGCTTGCCCAGGTAGACCTGTCGGGCCCTC 1162
A  K  K  END                                                                                                              323

CCTTCTCCATTTTGATTATTGCTGGCACTTGCTGGTTATAGCAGCAAGTGAAGCATTTCATTCTCAAGAAAGCGTTTGTTTTTGTTTGTTTTTAACCTCTGTTCCCTTTGTGGACAGCTC 1282

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TCACTGTCTCATCCTGTACAAACTAGTGTCTGCAGCTGTGGCAGCAGGAGCAACCTGCCACCCTGCCACCCACACTGCCCAGGCTGTCTGAAGCACACTCCCTCCCAGTGCACACTTAAC 1642
                                                                                                                  oligo 1
TGACGATTAAAGCCATTCTTTTCAATGTCTGTGATTCCTTCCTAAAGCCAAAGTTTCTGTTGGACTGTATTGGCACACCCTGGACATGAGGTGGCCAGGGCACCAAGGCTGCTGGCACAG 1762

GCCGCCTCCGTGGGGACTCAGAAAGAAGCAGGTTATTTTAACTAGCAATAGTGATGTAGTGCTGGGTAAGCTATTAATGGTGGAAGTTAATGAGACATTGTACAGTGCCCATATAGTCTA 1882

TTCACTGAGTAATCTTTTTACAGTTGGATCAGGCCTGAACCCGTCCATTCAGAAAGCTTCAAATTATAGAAACAACACTGTCCTATACGAGTGATCGATAATGCTTTCTTTGGCTACATT 2002

CTTTATTCTGCGGTGACATTGAGGCTTATAAATCAAAAGGAACTAACTTGCCGTCCACCGGTTTATACAGAACTCACAGTATCTATGACTTTTTTAAACTACGACCTGTTAAAATGAATC 2122
                                  oligo 2
TGTTTCCACAGATGCCGTGTACAATGCCATGTGCTAAGAATGATTTCAGACTTATTAAATGCGAGCTTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                  2224
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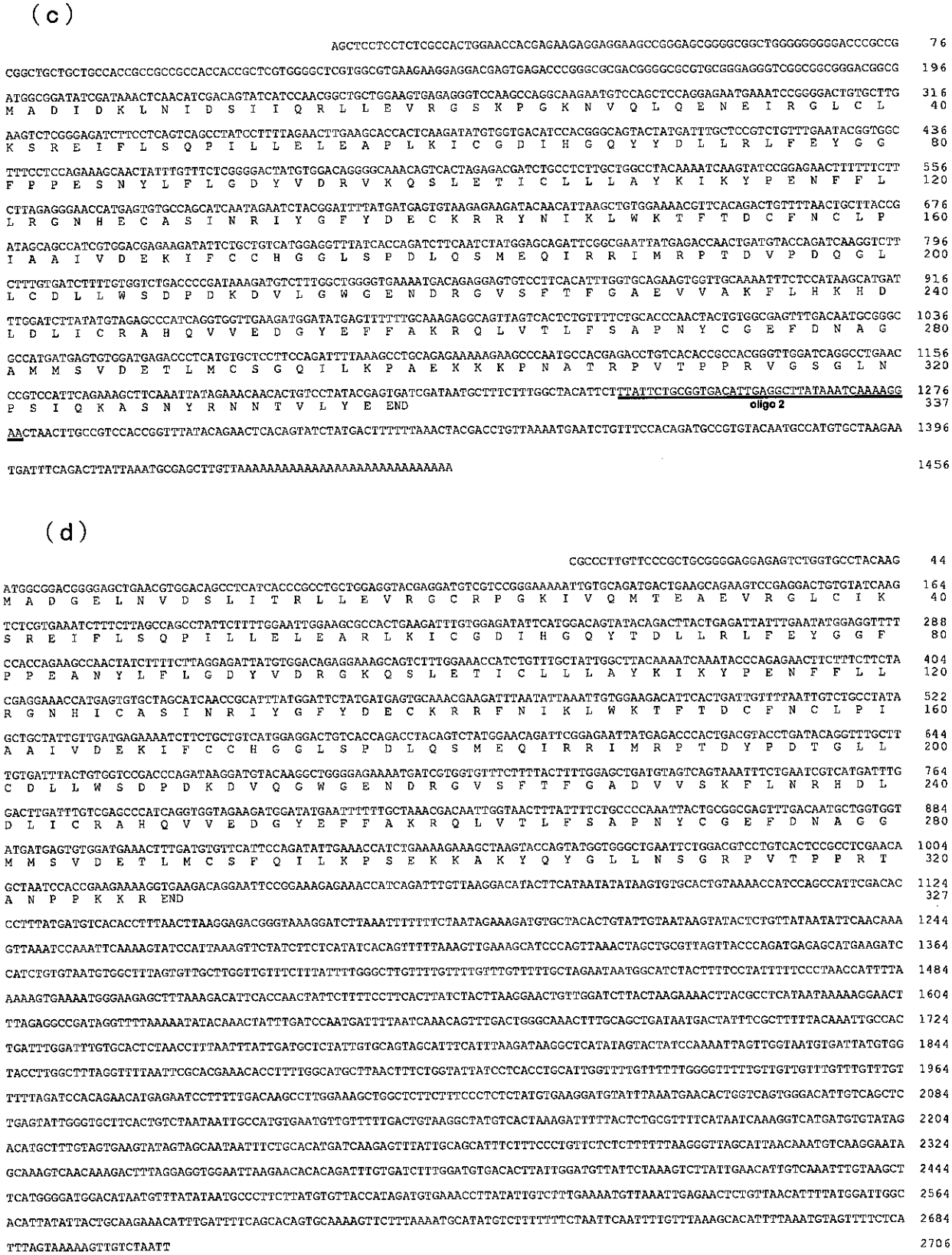
Fig. 2. Nucleotide and predicted amino acide sequences of cDNA clones for rat PP-1 catalytic subunits. a; *PP-1α*. b; *PP-1γ1*. The regions complementary to the synthetic oligomers used as probes are underlined. c; *PP-1γ2*. d; *PP-1δ*.


(c)
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ATGGCGGATATCGATAAACTCAACATCGACAGTATCATCCAACGGCTGCTGGAAGTGAGAGGGTCCAAGCCAGGCAAGAATGTCCAGCTCCAGGAGAATGAAATCCGGGGACTGTGCTTG 316
M A D I D K L N I D S I I Q R L L E V R G S K P G K N V Q L Q E N E I R G L C L 40
AAGTCTCGGGAGATCTTCCTCAGTCAGCCTATCCTTTTAGAACTTGAAGCACCACTCAAGATATGTGGTGACATCCACGGGCAGTACTATGATTTGCTCCGTCTGTTTGAATACGGTGGC 436
K S R E I F L S Q P I L L E L E A P L K I C G D I H G Q Y Y D L L R L F E Y G G 80
TTTCCTCCAGAAAGCAACTATTTGTTTCTCGGGGACTATGTGGACAGGGGCAAACAGTCACTAGAGACGATCTGCCTCTTGCTGGCCTACAAAATCAAGTATCCGGAGAACTTTTTTCTT 556
F P P E S N Y L F L G D Y V D R V K Q S L E T I C L L L A Y K I K Y P E N F F L 120
CTTAGAGGGAACCATGAGTGTGCCAGCATCAATAGAATCTACGGATTTTATGATGAGTGTAAGAGAAGATACAACATTAAGCTGTGGAAAACGTTCACAGACTGTTTTAACTGCTTACCG 676
L R G N H E C A S I N R I Y G F Y D E C K R R Y N I K L W K T F T D C F N C L P 160
ATAGCAGCCATCGTGGACGAGAAGATATTCTGCTGTCATGGAGGTTTATCACCAGATCTTCAATCTATGGAGCAGATTCGGCGAATTATGAGACCAACTGATGTACCAGATCAAGGTCTT 796
I A A I V D E K I F C C H G G L S P D L Q S M E Q I R R I M R P T D V P D Q G L 200
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L C D L L W S D P D K D V L G W G E N D R G V S F T F G A E V V A K F L H K H D 240
TTGGATCTTATATGTAGAGCCCATCAGGTGGTTGAAGATGGATATGAGTTTTTTGCAAAGAGGCAGTTAGTCACTCTGTTTTCTGCACCCAACTACTGTGGCGAGTTTGACAATGCGGGC 1036
L D L I C R A H Q V V E D G Y E F F A K R Q L V T L F S A P N Y C G E F D N A G 280
GCCATGATGAGTGTGGATGAGACCCTCATGTGCTCCTTCCAGATTTTAAAGCCTGCAGAGAAAAAGAAGCCCAATGCCACGAGACCTGTCACACCGCCACGGGTTGGATCAGGCCTGAAC 1156
A M M S V D E T L M C S G Q I L K P A E K K K P N A T R P V T P P R V G S G L N 320
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P S I Q K A S N Y R N N T V L Y E END 337
oligo 2
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TGATTTCAGACTTATTAAATGCGAGCTTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1456
(d)
CGCCCTTGTTCCCGCTGCGGGGAGGAGAGTCTGGTGCCTACAAG 44
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M A D G E L N V D S L I T R L L E V R G C R P G K I V Q M T E A E V R G L C I K 40
TCTCGTGAAATCTTTCTTAGCCAGCCTATTCTTTTGGAATTGGAAGCGCCACTGAAGATTTGTGGAGATATTCATGGACAGTATACAGACTTACTGAGATTATTTGAATATGGAGGTTTT 288
S R E I F L S Q P I L L E L E A R L K I C G D I H G Q Y T D L L R L F E Y G G F 80
CCACCAGAAGCCAACTATCTTTTCTTAGGAGATTATGTGGACAGAGGAAAGCAGTCTTTGGAAACCATCTGTTTGCTATTGGCTTACAAAATCAAATACCCAGAGAACTTCTTTCTTCTA 404
P P E A N Y L F L G D Y V D R G K Q S L E T I C L L L A Y K I K Y P E N F F L L 120
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R G N H I C A S I N R I Y G F Y D E C K R R F N I K L W K T F T D C F N C L P I 160
GCTGCTATTGTTGATGAGAAAATCTTCTGCTGTCATGGAGGACTGTCACCAGACCTACAGTCTATGGAACAGATTCGGAGAATTATGAGACCCACTGACGTACCTGATACAGGTTTGCTT 644
A A I V D E K I F C C H G G L S P D L Q S M E Q I R R I M R P T D Y P D T G L L 200
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C D L L W S D P D K D V Q G W G E N D R G V S F T F G A D V V S K F L N R H D L 240
GACTTGATTTGTCGAGCCCATCAGGTGGTAGAAGATGGATATGAATTTTTTGCTAAACGACAATTGGTAACTTTATTTTCTGCCCCAAATTACTGCGGCGAGTTTGACAATGCTGGTGGT 884
D L I C R A H Q V V E D G Y E F F A K R Q L V T L F S A P N Y C G E F D N A G G 280
ATGATGAGTGTGGATGAAACTTTGATGTGTTCATTCCAGATATTGAAACCATCTGAAAAGAAAGCTAAGTACCAGTATGGTGGGCTGAATTCTGGACGTCCTGTCACTCCGCCTCGAACA 1004
M M S V D E T L M C S F Q I L K P S E K K A K Y Q Y G L L N S G R P V T P P R T 320
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A N P P K K R END 327
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CATCTGTGTAATGTGGCTTTAGTGTTGCTTGGTTGTTTCTTTATTTTGGGCTTGTTTTGTTTTGTTTGTTTTTGCTAGAATAATGGCATCTACTTTTCCTATTTTTCCCTAACCATTTTA 1484
AAAAGTGAAAATGGGAAGAGCTTTAAAGACATTCACCAACTATTCTTTTCCTTCACTTATCTACTTAAGGAACTGTTGGATCTTACTAAGAAAACTTACGCCTCATAATAAAAAGGAACT 1604
TTAGAGGCCGATAGGTTTTAAAAATATACAAACTATTTGATCCAATGATTTTAATCAAACAGTTTGACTGGGCAAACTTTGCAGCTGATAATGACTATTTCGCTTTTTACAAATTGCCAC 1724
TGATTTGGATTTGTGCACTCTAACCTTTAATTTATTGATGCTCTATTGTGCAGTAGCATTTCATTTAAGATAAGGCTCATATAGTACTATCCAAAATTAGTTGGTAATGTGATTATGTGG 1844
TACCTTGGCTTTAGGTTTTAATTCGCACGAAACACCTTTTGGCATGCTTAACTTTCTGGTATTATCCTCACCTGCATTGGTTTTGTTTTTTGGGGTTTTTGTTGTTGTTTGTTTGTTTGT 1964
TTTTAGATCCACAGAACATGAGAATCCTTTTTGACAAGCCTTGGAAAGCTGGCTCTTCTTTCCCTCTCTATGTGAAGGATGTATTTAAATGAACACTGGTCAGTGGGACATTGTCAGCTC 2084
TGAGTATTGGGTGCTTCACTGTCTAATAATTGCCATGTGAATGTTGTTTTTGACTGTAAGGCTATGTCACTAAAGATTTTTACTCTGCGTTTTCATAATCAAAGGTCATGATGTGTATAG 2204
ACATGCTTTGTAGTGAAGTATAGTAGCAATAATTTCTGCACATGATCAAGAGTTTATTGCAGCATTTCTTTCCCTGTTCTCTCTTTTTTAAGGGTTAGCATTAACAAATGTCAAGGAATA 2324
GCAAAGTCAACAAAGACTTTAGGAGGTGGAATTAAGAACACACAGATTTGTGATCTTTGGATGTGACACTTATTGGATGTTATTCTAAAGTCTTATTGAACATTGTCAAATTTGTAAGCT 2444
TCATGGGGATGGACATAATGTTTATATAATGCCCTTCTTATGTGTTACCATAGATGTGAAACCTTATATTGTCTTTGAAAATGTTAAATTGAGAACTCTGTTAACATTTTATGGATTGGC 2564
ACATTATATTACTGCAAGAAACATTTGATTTTCAGCACAGTGCAAAAGTTCTTTAAAATGCATATGTCTTTTTTTCTAATTCAATTTTGTTTAAAGCACATTTTAAATGTAGTTTTCTCA 2684
TTTAGTAAAAAGTTGTCTAATT 2706

found in the same rat liver cDNA library. These five clones all gave identical restriction maps that differed from those of clones 1 and 2. The restriction map of the clone with the longest sequence of 1.4 kb, clone 3, is also shown in Fig. 1.

Five cDNA clones were obtained from a rat testis cDNA library, using a cDNA fragment of *dis2*ml as a probe. These five clones gave identical restriction maps that differed from that of clone 1. The map of clone 4, with the longest sequence of 1.5 kb is shown in Fig. 1.

Nucleotide sequences and deduced amino acid sequences

Nucleotide sequences were determined according to the strategy shown in Fig. 1. Clone 3 encoded a peptide of 330 amino acids, as rabbit *PP-1α* did,[7,8] and its amino acid sequence was identical with that of rabbit *PP-1α* product (Fig. 2a). The identity of clone 3 with rabbit *PP-1α* in the nucleotide sequences of their coding region was 89%. From these findings, we named clone 3 rat *PP-1α*. Rabbit *PP-1α* and *PP-1β* may be derived from the same gene but these proteins were different at the N-termini.[8] No rat clone homologous to rabbit *PP-1β* could be obtained.

Clone 1 was found to encode a peptide of 323 amino acids although *dis2*ml[9] encodes a peptide of 339 amino acids. The identity of the deduced amino acid sequences of the two was 95%. The deduced amino acid sequences of the two differed only in their carboxy-terminal regions: their amino acid sequences from position 1 to 314 were identical, but their amino acid sequence downstream from position 315 were completely different. The homologies of the predicted amino acid sequence of clone 1 and those of rabbit *PP-X* and *PP-Z*, and *Drosophila PP-V* and *PP-Y* were much less, 43%, 62%, 41% and 59%, respectively. These results suggested that clone 1 was a homologue of *dis2*ml. Therefore we named clone 1 *PP-1γ1* (Fig. 2b). We isolated another cDNA clone, *PP-1γ2*, which was derived from the same gene (see below). The identity of the nucleotide sequences of the coding regions of *PP-1γ1* and *dis2*ml was 93%.

Clone 2 encoded a peptide of 327 amino acids with an identical deduced amino acid sequence to that of *dis2*m2.[9] The identity of the nucleotide sequences of the coding regions of the two was 97%. Thus clone 2 is clearly a homologue of *dis2*m2, but not of *PP-1α*, *PP-1β*, *PP-1γ1*, *PP-V*, *PP-X*, *PP-Y* or *PP-Z*. Based on these findings, we named clone 2 *PP-1δ* (Fig. 2d).

Clone 4 encoded a 337 amino acid peptide with an identical amino acid sequence to that of mouse *dis2*ml. The identity of the nucleotide sequences of the coding regions of clone 4 and *dis2*ml was 98%. The nucleotide sequence of the region upstream of nucleotide 1139 in clone 4 (Fig. 2c) was identical with that upstream of nucleotide 1025 in clone 1 (Fig. 2b) and the downstream region from nucleotide 1140 in clone 4 was also identical with that from nucleotide 1906 of clone 1. These findings suggest that these clones were derived from the same gene by alternative splicing. Accordingly, we named clone 4 *PP-1γ2* (Fig. 2c). The difference in the deduced amino acid sequences of PP-1γ1 and PP-1γ2 was in the region downstream of amino acid 315, as was the difference between those of PP-1γ1 and dis2ml. Details of the difference will be discussed later.

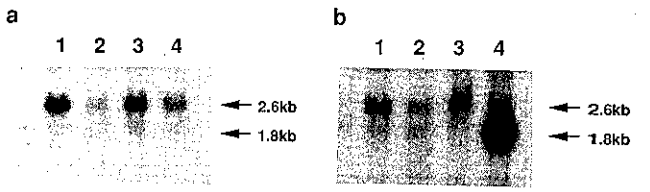

Fig. 3. Differential hybridization using synthetic oligonucleotides as probes. Samples of 10 μg of total RNA from normal rat tissues were blotted and hybridized with end-labeled synthetic oligo 1 (a) or oligo 2 (b). Lane 1, kidney; lane 2, heart; lane 3, brain; lane 4, testis

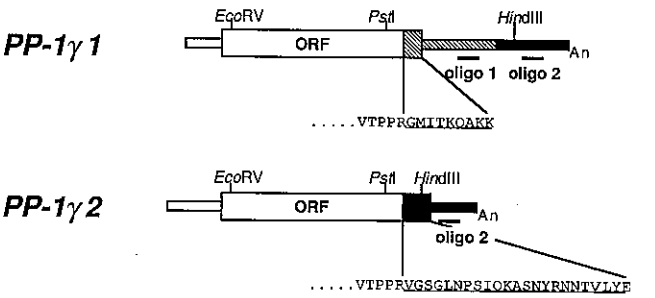

Fig. 4. Relationship between *PP-1γ1* and *PP-1γ2*. Regions shown by open boxes in *PP-1γ1* and *pp-1γ2* are identical. Regions shown by closed boxes in *PP-1γ1* and *PP-1γ2* have the same sequence. The hatched box indicates the region present only in *PP-1γ1*. The predicted amino acid sequences that differ in PP-1γ1 and PP-1γ2 are underlined. The regions corresponding to the oligo probes used in Fig. 3 are also indicated.

***PP-1γ1* and *PP-1γ2* produced by alternative splicing**

Differential hybridization was performed with the synthetic oligomers, oligo 1 and oligo 2 (Fig. 2b and 2c), as probes. Oligo 1 detected only 2.6 kb mRNA while oligo 2 detected both 2.6 kb and 1.8 kb mRNA (Fig. 3). This finding suggested that *PP-1γ1* corresponded to 2.6 kb mRNA, and *PP-1γ2* to 1.8 kb mRNA.

The relationship between *PP-1γ1* and *PP-1γ2* is shown in Fig. 4. The open and closed boxes in the maps of *PP-1γ1* and *PP-1γ2* show the two regions of identical nucleotide sequences. In *PP-1γ1*, there is an additional sequence shown as a hatched box (Fig. 4). In *PP-1γ2*, the

closed box includes part of its coding region but the same sequence in *PP-1γ1* corresponds to 3′non-coding region.

Comparison among protein phosphatases Comparison of these four cDNA clones revealed that the identities of the nucleotide sequences of these coding regions were in the range of 70–75% (Table I). The deduced amino acid sequences of these four cDNA clones were also highly conserved, their identities being 87–95% (Table I). On the other hand, the identities of the catalytic subunits of subgroups of PP-1 and PP-2 are less than 45%,[8] the highest identity of 45% being found between rat *PP-1γ1* and rat *PP-2Aβ*. Thus, these findings showed that the cDNA clones isolated in this study encoded isotypes of PP-1 catalytic subunits.

Table I. Identities of Nucleotide and Deduced Amino Acid Sequences of Catalytic Subunits of Rat PP-1

	Identity (%)			
	PP-1α	*PP-1γ1*	*PP-1γ2*	*PP-1δ*
PP-1α		76.0	73.7	75.0
PP-1γ1	92.5		—	72.0
PP-1γ2	89.7	95.2		70.1
PP-1δ	90.1	89.5	87.0	

The upper half shows the identities of nucleotide sequences, and the lower half those of the deduced amino acid sequences. The identity of the nucleotide sequences of *PP-1γ1* and *PP-1γ2* was not calculated, because those sequences are thought to be produced by alternative splicing.

The primary structures of the deduced amino acid sequences of rat PP-1α, PP-1γ1, PP-1γ2 and PP-1δ were compared (Fig. 5). The amino acid sequences present upstream of position 301 in PP-1α were highly conserved in all isotypes of the PP-1 catalytic subunits that encoded by cDNAs we cloned, differences mainly being present in their carboxy-terminals. At the same time, the deduced amino acid sequences of catalytic subunits of rat PP-1 were compared with those of rat PP-2A (Fig. 5), because the homology between the catalytic subunits of PP-1 and PP-2A has been reported to be high.[3] The central regions of these PP catalytic subunits are partially conserved and their amino acid sequence differences are mainly in their amino- and carboxy-terminal regions.

Conserved amino acids in PP-1 and PP-2 We compared the similarities of all the catalytic subunits of PPs so far reported (Fig. 6). Each isotype of catalytic subunits was represented by only one species, because the amino acid

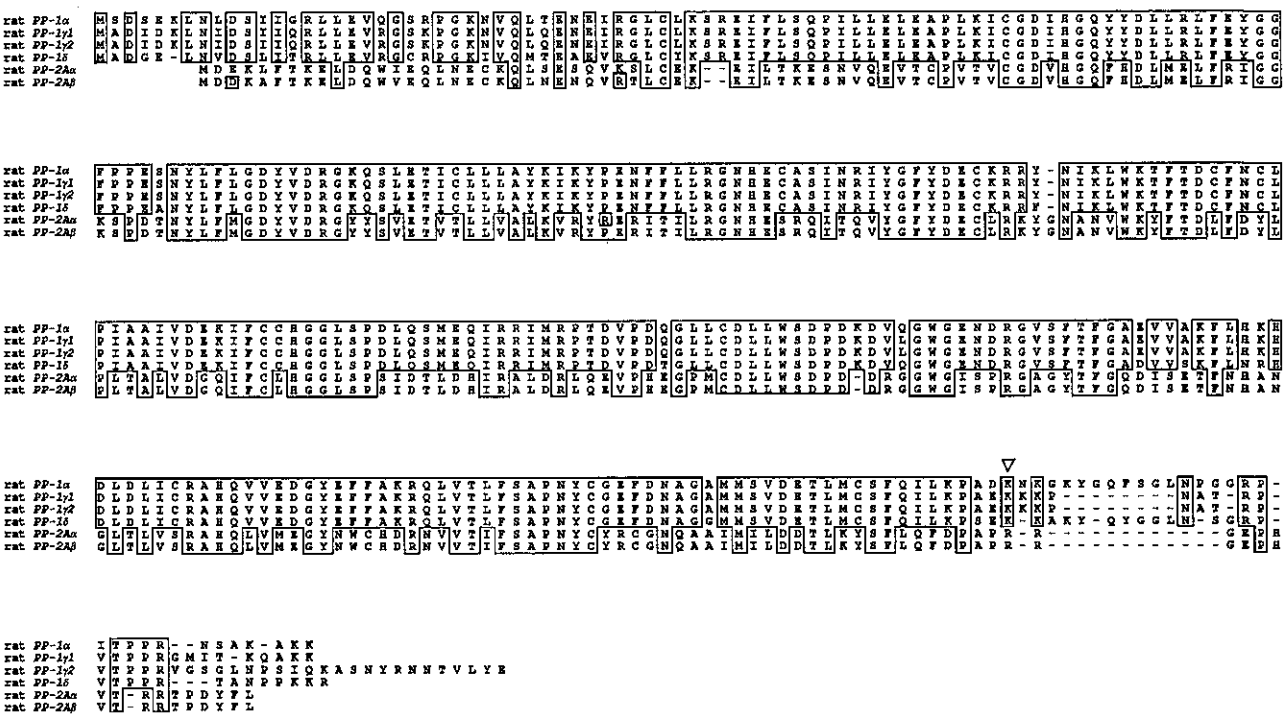

Fig. 5. Comparison of the amino acid sequences of catalytic subunits of rat PP-1 and PP-2A. *PP-1α*, *PP-1γ1* and *PP-1δ* were isolated from a rat liver cDNA library, and *PP-1γ2* was from a rat testis cDNA library. *PP-2Aα* and *PP-2Aβ* were from a rat liver cDNA library as reported previously. The amino acid sequences that are identical in the isotypes of PP-1 catalytic subunits are boxed. An arrowhead denotes the amino acid position 301 of rat PP-1α.

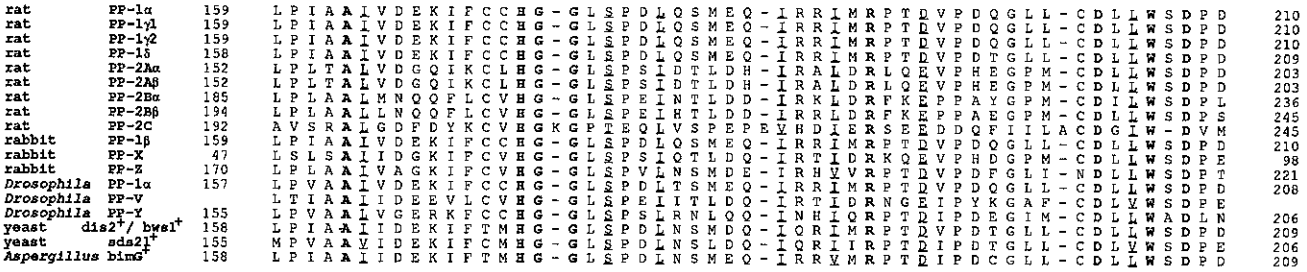

Fig. 6. Conserved sequences of the protein phosphatase family. Homologous sequences were searched for on rat PP-1α, PP-1γ1, PP-1γ2 and PP-1δ (this study), rat PP-2Aα and PP-2Aβ,[12, 13] rat PP-2Bα,[14] PP-2Bβ,[15] rat PP-2C,[32] rabbit PP-1β,[6] PP-X,[10] PP-Z[18] and *Drosophila* PP-1α, PP-V and PP-Y.[18, 20, 37] The genes *dis2*$^+$, *sds21*$^+$, *bws1*$^+$ and *bim*G were reported by Ohkura *et al.*,[9] Booher and Beach,[36] and Doonan and Morris.[38] Bold letters denote amino acid identity; underlined letters denote conservative amino acid substitution.

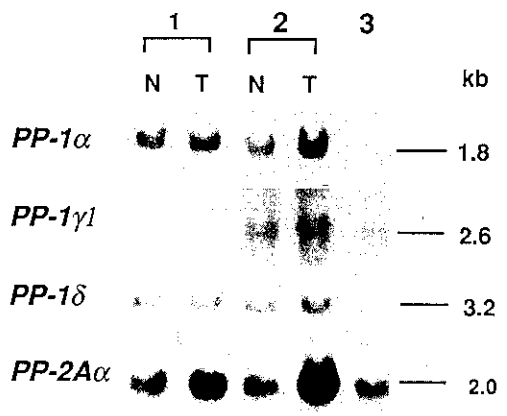

Fig. 7. Expression of *PP-1α*, *PP-1γ1*, *PP-1δ* and *PP-2Aα* in hepatocellular carcinomas induced by MeIQx. RNAs were extracted from nontumorous parts (N) and tumors (T) of the livers of two rats, No. 1 and No. 2. 1, the liver of No. 1 rat; 2, the liver of No. 2 rat; 3, the liver of a normal rat at the age of 45 weeks.

sequences of catalytic subunits are highly conserved in different species. We compared the amino acid sequences of 18 subunits, 9 from rats, 3 from rabbits, 3 from *Drosophila*, 2 from yeast and 1 from *Aspergillus*. As can be seen in Fig. 6, we found a conserved central region where several amino acid residues are identical in all 18 catalytic subunits (bold letters) and there are several conservative changes (underlined). In other regions than the central region, however, we found no amino acid sequence common to PP-2C and other PP catalytic subunits. The middle regions of all of the catalytic subunits of PP-1 and PP-2, as shown in Fig. 6, may thus play a key role in the function of phosphatase.

Expression of PP-1α, PP-1γ and PP-1δ in liver tumors

Expression levels of mRNA of *PP-1α*, *PP-1γ* and *PP-1δ* in two independent rat hepatocellular carcinomas induced by MeIQx were examined (Fig. 7). Level of *PP-1α* mRNA was increased about 2 times in both of the hepatocellular carcinomas as compared with the normal portion of livers of rats bearing the carcinomas, or the liver of a control rat. In contrary, almost no changes in the level of *PP-1γ1* and *PP-1δ* were observed. Of course no increase of *PP-1γ2* was found (data not shown). Thus *PP-1α* seems to be involved in hepatocarcinogenesis or hepatic cell proliferation. Expression of *PP-2Aα* mRNA was also examined as a positive control,[12, 13] and levels of *PP-2Aα* mRNA were found to be increased 3 and 5 times in these two tumors (Fig. 7), as expected.

DISCUSSION

We demonstrated that the encoded amino acid sequences of rat *PP-1α*, *PP-1γ2* and *PP-1δ* were identical with those of rabbit *PP-1α*, mouse *dis2*ml and mouse *dis2*m2, respectively. This is the first report of *PP-1γ1*, which encodes a new isotype of PP-1 catalytic subunit in rat. The amino acid sequences of amino-terminal and central regions of all the PP-1 isotypes that we cloned were highly conserved (Fig. 5), their differences being mainly in the carboxy-terminal regions. This finding suggests that this conserved region is involved in the enzyme activity and the carboxy-terminal region is not involved in enzyme activity, but plays some other role, such as in regulating the substrate specificity or binding of the catalytic subunits to specific regulatory subunits.

The results of nucleotide sequence analysis suggested that two cDNA clones, *PP-1γ1* and *PP-1γ2*, were produced by alternative splicing. This alternative splicing causes the difference in the amino acid sequences of their carboxy termini. *PP-1γ2* is highly expressed as a major species in germ cells, but is a minor species in somatic tissues in rats.[28] Thus, the *PP-1γ2* protein may have an important role in germ cells. Rabbit *PP-1α* and *PP-1β* are

also reported to be produced by alternative splicing which results in a difference in their predicted amino-terminal amino acid sequences.[8] So far we have not succeeded in isolating *PP-1β* from rat liver and testis cDNA libraries.

The mRNAs of some of the genes are shorter in the testis than in somatic tissues. Previously we found that *PP-2Aβ*, which encodes an isotype of the PP-2A catalytic subunit, expressed shorter mRNA in testis.[28] This shorter mRNA of *PP-2Aβ* is produced by alternative poly(A) addition.[29] A similar mechanism has been reported to be involved in the production of shorter mRNA of human *RIα* encoding the regulatory subunit of cAMP-dependent protein kinase.[30] Production of shorter mRNA for the human α-tubulin gene was found to be due to the use of a testis-specific promoter.[31] The encoded protein products, PP-2Aβ, RIα and α-tubulin in the testis are, however, the same as those in somatic tissues, unlike the PP-1γ1 and PP-1γ2 catalytic subunits.

We compared the amino acid sequences of almost all known PP catalytic subunits. No similarity was reported between PP-2C catalytic subunit and those of PP-1/PP-2A[3, 32] We found, however, that several amino acids are exactly conserved in all subunits derived from rat, rabbit, *Drosophila*, yeast and *Aspergillus*, as shown in Fig. 6. This finding suggested that this conserved region plays some important functional role. But these conserved amino acid sequences are not found in rat alkaline phosphatase[33] or human phosphotyrosine phosphatases.[34, 35] Therefore, we suggest that these conserved amino acid sequences compose the functional domain in PPs that recognizes phosphoserine and phosphothreonine residues.

With regard to the physiological role of PP-1, studies on cell cycle mutants of yeast have shown that PP-1 catalytic subunits are involved in mitotic initiation and mitotic disjunction.[9, 36] In mammalian cells, PP-1 is known to be involved in dephosphorylation of the ribosomal S6 protein, myosin P-light chain, rate-limiting enzymes of glycogen metabolism, and so on.[3] Different types of PP-1 catalytic subunits may have different abilities to bind specific regulatory subunits that may regulate substrate specificity. Changes of mRNA levels of these PP-1 catalytic subunits in rat hepatomas were specifically observed with *PP-1α*, but not with *PP-1γ1* or *PP-1δ*. We propose that the PP-1α protein would be involved in hepatocarcinogenesis and/or cell proliferation.

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