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MOLECULAR CLONING AND CHROMOSOMAL MAPPING OF THE HUMAN GENE FOR THE TESTIS-SPECIFIC CATALYTIC SUBUNIT OF CALMODULIN-DEPENDENT PROTEIN PHOSPHATASE (CALCINEURIN A)

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SUMMARY: A cDNA for an alternatively spliced variant of the testis-specific catalytic subunit of calmodulin dependent protein phosphatase (CaM-PrP) was cloned from a human testis library. The nucleotide sequence of 2134 base pairs (bp) encodes a protein of 502 amino acids (Mr ≈57,132) and pI 7.0. The cDNA sequence differs from the murine form of this gene by a 30 bp deletion in the coding region, the position of which matches those in the two other genes for the catalytic subunit. These data indicate that this alternative splicing event arose prior to the divergence of the three genes. The deduced sequence of the human protein is only 88% identical to the homologous murine form, in striking contrast to the other two CaM-PrP catalytic subunits which are highly conserved between mouse and human (≈99%); this indicates a more rapid rate of evolution for the testis-specific gene. Analysis of Southern blots containing DNA from human-hamster somatic cell hybrids show that the gene is on human chromosome 8.

Calmodulin-dependent protein phosphatase (PP2B, calcineurin) appears to be involved in a wide spectrum of biological activities, acting as a Ca^{2+}-dependent modifier of phosphorylation status (1,2). In some cases, such as epinephrine-induced glycogen breakdown in muscle (3), it may act in opposition to cAMP phosphorylation cascades by initiating broad-based phosphatase activity through dephosphorylation of inhibitors of PP1 (4). In testis, the motility of the sperm is thought to be controlled by cAMP-dependent phosphorylation (5) and a unique form of PP2B appears to be associated with the flagellum (6). Thus, it seems plausible that this phosphatase may alter cAMP regulated phosphorylation here as well, although perhaps through different mechanisms than in muscle.

The holoenzyme is composed of catalytic and regulatory subunits (60 and 18 kDa, respectively). To date, three distinct genes have been cloned for the catalytic subunit in mammals (7-13). Two of the three genes (*PP2Bα1* and *PP2Bα2*, or "α" and "β" forms) are highly expressed in brain and alternatively-spliced variants are known (8,9,14). These genes have been identified in humans, mice and rats, and are highly conserved between species (99% amino acid identity).

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In contrast, expression of a third gene (*PP2Bα3*, or "γ") is essentially testis-specific, and has been reported only in mice (10). The testis isoform is regulated developmentally, showing an apparent correspondence with the maturation of germ cells. This paper reports the cloning and characterization of an alternatively-spliced form of human *PP2Bα3* and documents its localization on human chromosome 8.

MATERIALS AND METHODS

Materials: Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 DNA ligase and reagents for DNA sequencing, using a modified form of T7 DNA polymerase (Sequenase) were from United States Biochemical. All components used for polymerase chain reaction (PCR) were obtained from Perkin-Elmer/Cetus and oligonucleotide primers were synthesized with a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novato, CA). Southern blots containing Eco RI-digested DNA from human-hamster hybrid cell lines were obtained from Bios (New Haven, CT), as were Hybond nylon membranes used for the screening of a phage library.

Cloning of the human cDNA: Template for the hybridization probe was prepared by PCR amplification of the open reading frame (ORF) of a murine testis clone (10); after purification of the PCR fragment, it was biotinylated by using random primers as described (15). A human testis cDNA library constructed in λgt11 (Clontech, Palo Alto, CA) was screened by plaque hybridization (8). After isolation of clones, phage DNA was purified from plate lysates by immunoaffinity procedures using LambdaSorb (Promega) and subcloned into pUC vectors as described (8). Plasmid DNA was prepared by the alkaline lysis method. DNA sequencing reactions for the longest clone, using the dideoxy termination method (16), were done twice on both strands using primers located ≈200 bp apart.

Southern blot analysis: The template for the hybridization probe was generated by PCR amplification of a 122-bp region in the human cDNA (see Fig. 1). A radiolabeled hybridization probe was prepared by primer extension of the template using both sense and anti-sense primers, essentially as described (8). The Southern blots containing chromosomal DNA were prehybridized for 4 hrs at 42°C in a solution containing 6x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 μg/ml salmon sperm DNA and 50% formamide. Hybridization was carried out for 15 hrs at 42°C in the same solution containing $1x10^6$ cpm of probe per ml of hybridization solution. The blots were then washed sequentially for 15-min periods with 2X SSC/0.1% SDS and 0.2X SSC/0.1% SDS at room temperature, followed by a final 15 min wash at 37°C in 0.1X SSC/0.1% SDS.

RESULTS AND DISCUSSION

Cloning of an alternatively spliced *PP2Bα3* cDNA from a human testis library:

A human testis library constructed in λgt-11 was screened using a biotinylated probe for the murine testis catalytic subunit (α3 isoform) (10); out of 40,000 plaques screened, 3 positive clones were found. All of these contain an identical ORF of 1509 bp coding for a protein of molecular mass, 57,132, that has a predicted isoelectric point of 7.0. The clone having the longest insert, HTα-1, possesses 286 bp of 5' untranslated region (UTR) and 339 bp of 3' UTR (Fig. 1), but lacks a region of polyadenylation. The nucleotide sequence of the human cDNA is highly homologous to the murine testis-specific form (≈85% identity), except that 30 base pairs are deleted in a region between the CaM-

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                                                                    -286 GGGCCACCCTTAGCAG CGGTCGCGGTCGGTGCCGAAGCGGTGTTCC -241
CCGCCTTAGCCGCTGCGCCTCCCAAGAGAG CGGCCGGTGGGCCCTCGTCCTGTCAGTGGC GTCGGAGGCCGGCCTGCGGTGGCCGCGCCC TTCTGGTGCTCGGACACCGCTGAGGAGCCG -121
GGGCCGGGCACGGCTGGCTGACGGCTCCGG GCAGCTAAGGCTGCCCGAGGAGAAGGCGGC GGCCGCGGCGTAGGCGCACGTCCGGCGGGC TCCTGGAGCCTGGAGGAGGCCGAGGGGACC -1

ATGTCCGGGAGGCGCTTCCACCTCTCCACC ACCGACCGCGTCATCAAAGCTGTCCCCTTT CCTCCAACCCAACGGCTTACTTTCAAGGAA GTATTTGAGAATGGGAAACCTAAAGTTGAT 120
M S G R R F H L S T  T D R V I K A V P F  P P T Q R L T F K E  V F E N G K P K V D  40

GTTTTAAAAAACCATTTGGTAAAGGAAGGA CGACTGGAAGAGGAAGTAGCCTTAAAGATA ATCAATGATGGGGCTGCCATCCTGAGGCAA GAGAAGACTATGATAGAAGTAGATGCTCCA 240
V L K N H L V K E G  R L E E E V A L K I  I N D G A A I L R Q  E K T M I E V D A P  80

ATCACAGTATGTGGTGATATTCATGGACAA TTCTTTGACCTAATGAAGTTATTTGAAGTT GGAGGATCACCTAGTAACACACGCTACCTC TTTCTGGGTGACTATGTGGACAGAGGCTAT 360
I T V C G D I H G Q  F F D L M K L F E V  G G S P S N T R Y L  F L G D Y V D R G Y  120

TTCAGTATAGAGTGTGTGCTGTATTTATGG AGTTTAAAGATTAATCATCCCAAAACATTG TTTCTGCTTCGGGGAAATCATGAATGCAGG CATCTTACAGACTATTTCACCTTCAAACAG 480
F S I E C V L Y L W S  L K I N H P K T L  F L L R G N H E C R  H L T D Y F T F K Q  160

GAATGTCGAATCAAATATTCGGAACAGGTG TATGATGCCTGTATGGAGACATTTGACTGT CTTCCTCTTGCTGCCCTCTTAAACCAGCAG TTTCTCTGTGTACATGGAGGAATGTCACCT 600
E C R I K Y S E Q V  Y D A C M E T F D C  L P L A A L L N Q Q  F L C V H G G M S P  200

GAAATTACTTCTTTAGATGACATTAGGAAA TTAGACAGGTTTACGGAACCTCCCGCCTTT GGACCTGTGTGTGACCTGCTTTGGTCTGAT CCCTCAGAGGATTATGGCAATGAGAAGACC 720
E I T S L D D I R K  L D R F T E P P A F  G P V C D L L W S D  P S E D Y G N E K T  240

TTGGAGCACTATACCCACAACACTGTCCGA GGGTGCTCTTATTTCTACAGTTACCCTGCA GTTTGTGAATTTTTGCAGAACAATAATTTA CTATCAATTATCAGAGCCCATGAAGCCCAA 840
L E H Y T H N T V R  G C S Y F Y S Y P A  V C E F L Q N N N L  L S I I R A H E A Q  280

GATGCTGGGTATCGAATGTACAGGAAGAGC CAAGCCACAGGCTTTCCATCACTTATTACA ATTTTCTCTGCCCCCAATTACCTAGATGTC TATAACAATAAAGCTGCTGTGTTGAAATAT 960
D A G Y R M Y R K S  Q A T G F P S L I T  I F S A P N Y L D V  Y N N K A A V L K Y  320

GAAAACAATGTCATGAATATCAGGCAGTTT AACTGTTCTCCACACCCCTACTGGCTTCCA AACTTTATGGATGTTTTCACATGGTCTTTG CCTTTTGTTGGGGAAAAAGTCACAGAGATG 1080
E N N V M N I R Q F  N C S P H P Y W L P  N F M D V F T W S L  P F V G E K V T E M  360

CTGGTAAATGTGCTCAACATATGCTCTGAT GACGAACTGATTTCTGATGATGAAGCAGAA GGAAGCACTACAGTTCGTAAGGAGATCATC AGGAATAAGATCAGAGCCATTGGGAAGATG 1200
L V N V L N I C S D  D E L I S D D E A E  G S T T V R K E I I  R N K I R A I G K M  400

GCACGGGTCTTTTCAATTCTTCGGCAAGAA AGTGAGAGTGTGCTGACTCTCAAGGGCCTG ACTCCCACAGGCACACTCCCTCTGGGCGTC CTCTCAGGAGGCAAGCAGACTATCGAGACA 1320
A R V F S I L R Q E  S E S V L T L K G L  T P T G T L P L G V  L S G G K Q T I E T  440

GCCATCAGAGGGTTCTCGCTTCAGCACAAG ATCCGGAGTTTTGAAGAAGCGCGAGGTCTG GACCGAATTAATGAGCGAATGCCACCCCGA AAGGATAGCATATACCCTGGTGGGCCAATG 1440
A I R G F S L Q H K  I R S F E E A R G L  D R I N E R M P P R  K D S I Y P G G P M  480

AAATCTGTAACCTCAGCACACTCACATGCT GCGCACAGGAGCGACCAAGGGAAGAAAGCC CATTCATGACTTAGAGTCCTGCCGTGCTCA GGTGGATCTAAAACTCAAGAACAAATTCTA 1560
K S V T S A H S H A  A H R S D Q G K K A  H S . 502

TTTATTTATTATTGGAAAATGAAAAGCAAC TCAAAACAACTTCAACCTGGAGGTGCATTT ATAATTCAGTCTGCATTTATTCTGTAAAAA GGTGACTGTTTTATAAATTCTTTTAATTTA 1680
TGTTCAATATATATAAAAAGTGCATCTGTT TTGTTTTTCCCTTTTTTCTCCATAATTTTA AGAAATGAATCTGATTGTTGTCAACACATT TGTGAAGTCTTGTGCTATAAAGGGGAACTT 1800
CCCCTAATAAAAGGGCCTTGGAAACCTCAA ACCTGGGTTTCTGACCCC  1848
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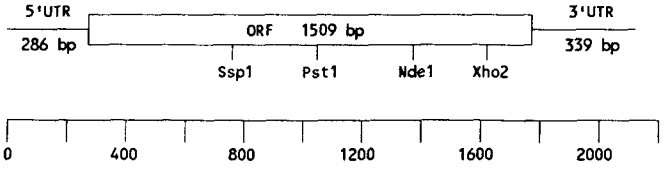

Figure 1. Nucleotide and deduced amino acid sequences for the catalytic subunit of CaM-PrP from human testis. The nucleotide and deduced amino acid sequences of the clone HTα-1 are presented, with positions in the 5' UTR represented as negative numbers. The underlined region (bp 271-392) represents the segment that was PCR-amplified and used in preparing the hybridization probe for Fig. 3. A map of unique restriction endonuclease sites is shown at the bottom of the figure. ORF, open reading frame.

binding (7) and autoinhibitory domains (17); this corresponds to the absence of amino acid 440-449 in the deduced mouse sequence (Fig. 2). Although it has been shown that homologous alternatively-spliced forms exist for the neural (α1, α2)

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Hum  MSGRRFHLSTTDRVIKAVPFPPTQRLTFKEVFENGKPKVDVLKNHLVKEGRLEEEVALKIINDGAAILRQ  70
Mur  ..V..PQF...E..........R...L..........M.L..........V.................K.  70

Hum  EKTMIEVDAPITVCGDIHGQFFDLMKLFEVGGSPSNTRYLFLGDYVDRGYFSIECVLYLWSLKINHPKTL 140
Mur  .......E........V..................................................... 140

Hum  FLLRGNHECRHLTDYFTFKQECRIKYSEQVYDACMETFDCLPLAALLNQQFLCVHGGMSPEITSLDDIRK 210
Mur  .............E..............M......H...........................C.E.... 210

Hum  LDRFTEPPAFGPVCDLLWSDPSEDYGNEKTLEHYTHNTVRGCSYFYSYPAVCEFLQNNNLLSIIRAHEAQ 280
Mur  ....S.................L....S...................F............S.......... 280

Hum  DAGYRMYRKSQATGFPSLITIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCSPHPYWLPNFMDVFTWSL 350
Mur  .........N.............................................................. 350

Hum  PFVGEKVTEMLVNVLNICSDDELISDDEAEGSTTVRKEIIRNKIRAIGKMARVFSILRQESESVLTLKGL 420
Mur  ..............I......E.MNVT..-..A..G...V.K..............TV..E...N..... 419

Hum  TPTGTLPLGVLSGGKQTIET----------AIRGFSLQHKIRSFEEARGLDRINERMPPRKDSIY--PGG 478
Mur  ....................AKQEAAEERE.....TIA.R.....................EAS.HHDA. 489

Hum  PMKSVT-SAHSHAAHRSDQGKKAHS* 502
Mur  R.H.HSHPP.PQ.SR.T.H....L* 513
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Figure 2. Comparison of the deduced amino acid sequences of cDNAs corresponding to human and murine isoforms of the testis-specific catalytic subunit, PP2Bα3. The deduced sequence of the human cDNA ("Hum") (this paper) is aligned with that of murine cDNA ("Mur") reported in reference 10; spaces needed to optimize homology are indicated by hyphens. The cumulative number of residues for each form is indicated on the right. Positions of identity are indicated by periods.

isoforms of the catalytic subunit (8,14), this is the first evidence for such a variant of the α3, or testis, isoform. This strongly suggests that alternative exon usage may give rise to molecular isoforms of the catalytic subunit in testis, as has been documented in brain (8). Because all three mammalian genes for the CaM-PrP catalytic subunit exhibit the same spliced exon, one can assume that an ancestral gene for this enzyme acquired the sequences for the alternative splicing prior to its divergence into the three genes. Further, there appears to be selective pressure for the maintenance of this molecular variant in all three genes. The functional significance of expression of this alternative exon is under study in our laboratory.

Chromosomal mapping of the human gene for *PP2Bα3*:

The genes for the "neural" CaM-PrP catalytic subunits, *PP2Bα1* and *PP2Bα2*, have been assigned to human chromosome 4 and 10, respectively (13), suggesting that their expression is not linked physically. Because it was of interest to determine if the gene for the testicular isoforms showed any obvious linkage with these or other genes, Southern blot analysis of DNA from somatic cell hybrids was carried out to make its human chromosomal assignment. The 122-bp probe used in this study was shown previously to lack cross-hybridization with the other two genes, under the conditions of stringency used (10). The majority of this probe sequence is contained within exon 3 of the *PP2Bα1* gene (S. Higuchi and R. L. K.,

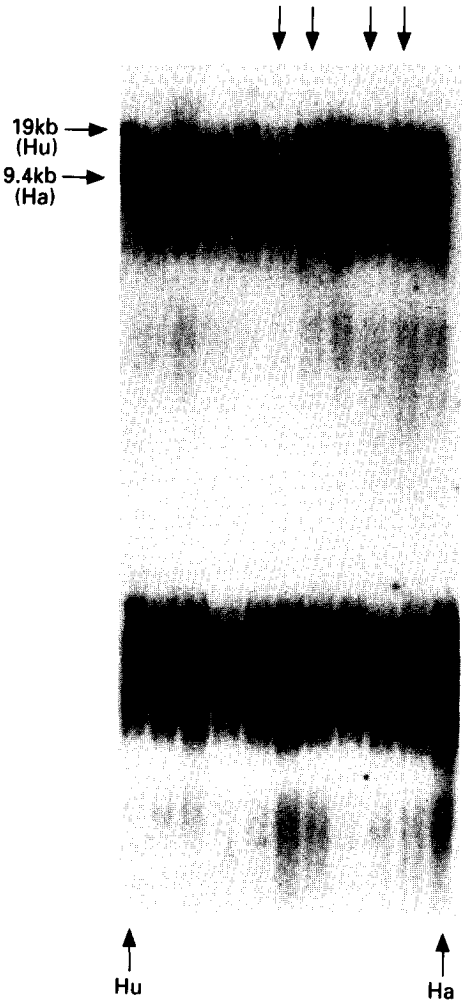

Figure 3. Assignment of the testis-specific catalytic subunit gene to a human chromosome by Southern blot analysis of DNA from human-hamster hybrid cell lines. Nylon membranes containing DNA from human-hamster hybrid cell lines were obtained from Bios Corporation, New Haven, CT. Each lane containing 5 μg of Eco RI-digested DNA. Samples were applied in two regions of the agarose gel (11 samples at the top and 11 samples in the middle) and, after electrophoresis, the DNA was transferred to nylon membranes. Lanes containing human (Hu) and hamster (Ha) DNA were included in control lanes at either end of the blots (see outside lanes). The blot was hybridized to ^{32}P-radiolabeled cDNA probe for the human PP2Bα3 (see Fig. 1). Hybridization in control lanes showed a 19 kb fragment that is specific for human (Hu) and a 9.4 kb fragment for the hamster (Ha) homologue. Hybrid cell lines which gave specific 19 kb fragments are indicated by arrows.

unpublished data) and the exon/intron boundaries of the testis gene determined so far coincide exactly with those of α1 (T. M. and R. L. K., unpublished data); thus, it seems likely that this area represents a conserved exon among the CaM-PrP catalytic subunit genes. As shown in Fig. 3, a specific 19 kb fragment, corresponding to the control sample of human DNA (lane 1), is present in four hybrid cell lines containing chromosome 8 (lanes 6,7,9,and 10) and is absent in

all hybrids lacking this chromosome. All lanes also show hybridization to a 9.4 kb EcoR1 fragment derived from the hamster genomic DNA. Because hybridization to the 19 kb fragment was never seen in samples derived from cells lacking this chromosome, these data demonstrate that the gene for the testis isoform of the CaM-PrP catalytic subunit resides on human chromosome 8.

Comparison of the deduced structures of human and murine PP2Bα3:

The overall deduced sequence of the human *PP2Bα3* catalytic subunit was 80% identical to the *PP2Bα1* human gene (8, and T. M. and R. L. K., unpublished data) and 81% identical to the *PP2Bα2* gene (9). Because of their similar sequence relatedness, this suggests that these proteins diverged from an ancestral gene at about the same time. For either of the two neural forms of the catalytic subunit, the deduced sequences of rodent and human forms are essentially the same (>99%) (7-9, 11,12). In contrast, however, the present study shows that the testis gene is much less conserved than the neural forms, with identity of murine and human sequences being only 88% (Fig. 2). Although substitutions are seen throughout the ORF, a region of 30 amino acids at the extreme carboxyl terminus displays striking differences. Interestingly, despite its divergence, this domain has a net positive charge and contains several histidines, properties that are similar to those in the murine form (10). The reduced sequence identity of the mammalian α3 catalytic subunits suggests that this isoform may be evolving rapidly, and implies that some aspect of tissue-specific function is less stringently conserved. Because it was suggested that the testis isoform may play a role in germ-cell maturation, and/or in the motility of sperm, the data presented here may suggest that species differences in the biochemistry or physiology of these events is best accommodated by a less constrained evolution of enzyme structure.

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