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CHROMOSOMAL MAPPING OF THE HUMAN GENES FOR THE CALMODULIN-DEPENDENT PROTEIN PHOSPHATASE (CALCINEURIN) CATALYTIC SUBUNIT*

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SUMMARY: Multiple catalytic subunits of the Ca^{2+} and calmodulin (CaM)-dependent protein phosphatase (PrP) ("calcineurin" or PrP-2B) are derived from at least two structural genes, type 1 ("calcineurin Aα") and type 2 ("calcineurin Aβ"), each of which can produce alternatively spliced transcripts. To examine the possible linkage of these genes, we analyzed genomic DNA from human/hamster hybrid cell lines using probes of 122 base pairs that were designed to bind selectively to exon 3 of the open reading frame. In this region, the nucleotide sequence of the type 2 murine cDNA that we cloned was >99% identical to the type 2 human cDNA but only 78% identical to the type 1 human cDNA. Hybridization to Southern blots containing DNA from all human chromosomes showed that gene 1 was found on chromosome 4, whereas gene 2 segregated to chromosome 10. These data suggest that expression of the two calcineurin genes is not physically linked.

The Ca^{2+} and calmodulin (CaM)-dependent phosphatases (PrP) represent a subset of enzymes (type 2B) within the larger superfamily of serine/threonine protein phosphatases (1). Their structures are distinct from those of the other major phosphatase forms, PrP-1 and PrP-2A, in that they have two subunits: a catalytic ("A") subunit of ≈ 60 kDa and a regulatory ("B") subunit of 19 kDa that binds Ca^{2+} and has high structural similarity with CaM (2).

The biological roles of this enzyme, of which there are neural and non-neural isoforms (3,4), are not yet completely understood, and it is likely that different tissues may use this activity in specific, Ca^{2+}-dependent signaling pathways. The phosphatase is highly concentrated in brain, where it was first isolated and named "calcineurin" (CN) (3). These forms may be needed for neuronal growth or neurotransmission inasmuch as their expression is strongly correlated with synaptogenesis (5,6). In skeletal muscle, this enzyme plays a major role in attenuating the breakdown of glycogen induced by epinephrine (7),

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through its dephosphorylation of Inhibitor-1, a heat-stable inhibitor of PrP-1. In lymphocytes, where CaM-PrP is the major soluble CaM-binding protein (8), recent studies suggest that immunosuppressant drugs such as cyclosporin A may exert their pharmacologic effects through this enzyme, by forming a complex with the cytoplasmic drug receptor that selectively binds to, and inhibits, this phosphatase (9). Also, a testicular form of this enzyme has been described that is tightly bound to sperm flagella and that may play a role in the motility of these cells (10).

Recent molecular biological studies have shown the presence of two distinct structural genes coding for the CN catalytic subunit in brain (11-15). In terms of protein abundance, the type 1 or "α" gene products appear to predominate over the type 2 or "β" forms (16,17), the latter containing a characteristic polyproline region on the amino terminus (13). Each of these genes can produce additional variants by alternative splicing, and a total of 5 unique cDNA isoforms have been described so far from rat, mouse and human libraries (12-15,18). Because it was of interest to determine the potential for physical linkage between these genes and to document their positions relative to genetic markers, we have compared their localization on human chromosomes.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones: In early studies, a 358-base pair (bp) cDNA clone (TA 3.2) with high homology to human type 2 cDNAs (13) was isolated when a mouse thymocyte library was screened with CNα-4, a type 1 brain cDNA (12). The insert from this clone was biotinylated by random-primed labeling and used to screen a mouse thymocyte cDNA library constructed in Uni-ZAP XR vector (Stratagene, La Jolla, CA); all details of probe preparation, hybridization, washing and secondary screening were as described previously (12). The insert-containing portions of phage DNA from positive clones were excised as phagemids in *E. coli* and phagemid DNA was purified using the alkaline lysis procedure (19). DNA sequencing was performed by the dideoxy nucleotide chain termination method (20); reactions for the longest clone (TCα-3) were done twice on both strands using primers located ≈200 bp apart.

Preparation of hybridization probes: Radiolabeled probes were prepared by primer extension, using the Klenow fragment of DNA polymerase I, of specific templates prepared by polymerase chain reaction (PCR); 18-nucleotide "sense" and "antisense" primers from the 5' and 3' ends of the template were used to produce the PCR fragments. The template for gene 1 was generated by PCR amplification of a 122 bp region from a human brain clone (CNHα-3) for the CN catalytic subunit; this corresponds to bps 283 to 404 of the open reading frame of the murine cDNA reported by Kincaid et al. (12). Template for gene 2 was generated from the mouse thymocyte cDNA clone (TCα-3) that is shown in Figure 1 (boxed area). These two templates have 78% nucleotide sequence identity, although they code for identical deduced amino acids (see Table 1). After purification of the PCR fragments by gel electrophoresis, probes were prepared as described (12) by radiolabeling with the Klenow fragment and both sense and antisense primers, using [^{32}P]dCTP and [^{32}P]dATP (3000 Ci/mmol; New England Nuclear, DuPont); specific activities were typically 1-2 x 10^9 cpm/μg of DNA.

Southern blot analysis: Southern blots containing *Eco* RI-digested DNA from 27 human-hamster hybrid cell lines were obtained from Bios Corporation (New Haven,

CT). These samples were distributed on two separate "panels", each of which contained DNA from 16 hybrid cell lines as well as 2 samples each of *Eco* RI-digested "control" DNAs (i.e. hamster and human). These hybrid cell lines were characterized for their chromosomal constitution by trypsin Giemsa banding and were also analyzed for the chromosomal mapping of known DNA markers. The derivation and characterization of these cell lines have been described (21).

Hybridization conditions: Southern blots were prehybridized for at least 4 hrs at 42°C in a solution containing 4X SSC, 5X Denhardt's solution, 0.2% SDS, 50 μg/ml yeast tRNA, 100 μg/ml herring sperm DNA and 50% (v/v) deionized formamide. Hybridization was carried out for 12-16 hrs at 42°C in the same solution containing 10% dextran sulfate (w/v) and 1 x 10^6 cpm of probe per ml of hybridization solution. The blots were then washed successively with 2X SSC, 0.1% SDS and 0.2X SSC, 0.1% SDS for 15 minutes each at room temperature followed by a final wash at 55°C in 0.1X SSC, 0.1% SDS for 15 minutes. The blots were exposed to Kodak XAR-5 film at -70°C with intensifying screens.

RESULTS AND DISCUSSION

To date, molecular isoforms of the catalytic subunit of calcineurin have been reported that apparently arise from exon splicing events in the two major genes (12,13). In the case of gene 1, a 30 bp fragment near the carboxyl terminus can be deleted by alternative splicing and transcripts for both "intact" and "deleted" forms have been demonstrated *in vivo* (12). This deletion also was observed in a gene 2 clone isolated from a carcinoma cell line (18). The fact that this variant is seen for both genes indicates that the alternative splicing event must have occurred in the ancestral gene before gene duplication. Another isoform of the type 2 gene, cloned from a human brain library, contains a 54 bp insertion in the catalytic domain and a shortened carboxyl terminus (13). Since a type 1 isoform comparable to this has not been reported, this splicing event may have occurred after gene duplication. In order to examine the chromosomal relationship of these two genes in humans, we compared DNA from somatic cell hybrids using, as probes, a type 1 human cDNA and a type 2 mouse cDNA (see below) that was essentially identical to the type 2 human form.

Cloning of a murine type 2 cDNA from a thymocyte library

Using a biotinylated cDNA probe, a total of 180,000 plaques were screened and 7 positive clones were isolated. Three cDNAs were greater than 1800 bp in length and were analyzed; the sequence of the clone containing the largest segment (1548 bp) of coding region (TCα-3) is shown in Fig. 1. The deduced amino acid sequence of the mouse cDNA differs from those of the rat (15) and human (13) at only 4 and 6 residues, respectively; except for the first 30 nucleotides, the open reading frame appears to be complete when compared to the human or rat cDNAs reported previously (13,15). The nucleotide sequence, which included 252 bp of 3' untranslated region, is 95% identical to the human and 97% identical to the rat homologues of calcineurin gene 2 (13,15). All murine thymocyte clones that were isolated lacked the initial coding and 5'

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CCGCCCCCGCCCCCGCCCCCACCGCCCCCT CTCGGGGCCGACCGCGTCGTCAAAGCTGTT CCTTTTCCCCCAACTCATCGGCTGACATCT GAAGAAGTGTTTGATATGGATGGGATACCC 120
P  P  P  P  P  P  P  P  P  P  L  G  A  D  R  V  V  K  A  V  P  F  P  P  T  H  R  L  T  S  E  E  V  F  D  M  D  G  I  P  40

AGGGTTGATGTTCTGAAGAACCATCTGGTA AAAGAAGGGCGGGTGGATGAAGAAATTGCA CTAAGAATTATCAATGAGGGTGCTGCCATA CTTCGGCGGGAGAAAACCATGATAGAAGTA 240
R  V  D  V  L  K  N  H  L  V  K  E  G  R  V  D  E  E  I  A  L  R  I  I  N  E  G  A  A  I  L  R  R  E  K  T  M  I  E  V  80

GAAGCTCCAATTACAGTGTGTGGTGACATC CATGGCCAATTTTTTGATCTGATGAAACTT TTTGAAGTAGGAGGATCACCTGCTAATACA CGATACCTTTTTCTTGGTGATTATGTGGAC 360
E  A  P  I  T  V  C  G  D  I  H  G  Q  F  F  D  L  M  K  L  F  E  V  G  G  S  P  A  N  T  R  Y  L  F  L  G  D  Y  V  D  120

AGAGGTTATTTTAGTATAGAGTGTGTCTTA TATTTATGGGTCTTGAAGATTCTATACCCA AGCACATTATTCCTTCTGAGAGGCAACCAT GAATGCAGACACCTTACTGAATATTTTACC 480
R  G  Y  F  S  I  E  C  V  L  Y  L  W  V  L  K  I  L  Y  P  S  T  L  F  L  L  R  G  N  H  E  C  R  H  L  T  E  Y  F  T  160

TTTAAGCAGGAATGTAAAATTAAATATTCA GAAAGAGTCTATGAAGCTTGTATGGAGGCT TTTGACAGCTTGCCCCTTGCTGCACTTCTA AACCAACAATTTCTTTGTGTTCATGGTGGA 600
F  K  Q  E  C  K  I  K  Y  S  E  R  V  Y  E  A  C  M  E  A  F  D  S  L  P  L  A  A  L  L  N  Q  Q  F  L  C  V  H  G  G  200

CTTTCACCAGAAATACACACACTGGATGAT ATTAGGAGATTAGATAGATTTAAAGAGCCA CCTGCATTTGGACCAATGTGTGACTTGCTA TGGTCTGATCCTTCTGAAGACTTTGGAAAT 720
L  S  P  E  I  H  T  L  D  D  I  R  R  L  D  R  F  K  E  P  P  A  F  G  P  M  C  D  L  L  W  S  D  P  S  E  D  F  G  N  240

GAAAAATCACAAGAACATTTTAGTCATAAT ACAGTTCGAGGATGTTCTTATTTTTATAAC TATCCAGCAGTGTGTGAATTTTTGCAAAAC AATAATTTGTTATCGATTATTAGAGCTCAT 840
E  K  S  Q  E  H  F  S  H  N  T  V  R  G  C  S  Y  F  Y  N  Y  P  A  V  C  E  F  L  Q  N  N  N  L  L  S  I  I  R  A  H  280

GAAGCTCAAGATGCAGGCTATAGAATGTAC AGAAAAAGTCAAACTACAGGGTTTCCTTCA TTAATAACAATTTTTTCGGCACCTAATTAC TTAGATGTCTACAATAATAAAGCTGCTGTA 960
E  A  Q  D  A  G  Y  R  M  Y  R  K  S  Q  T  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  320

CTAAAGTATGAAAATAATGTGATGAACATT CGACAGTTTAATTGCTCTCCACATCCTTAT TGGTTGCCCAATTTTATGGATGTCTTTACA TGGTCTTTACCATTTGTTGGAGAAAAAGTG 1080
L  K  Y  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  360

TACAGAAATGTTGGTAAATGTTCTGAGTATT TGTTCTGATGATGAACTAATGACAGAAGGT GAAGACCAGTTTGATGTAGGTTCAGCTGCA GCCCGGAAAGAAATCATAAGAAACAAGATC 1200
T  E  M  L  V  N  V  L  S  I  C  S  D  D  E  L  M  T  E  G  E  D  Q  F  D  V  G  S  A  A  A  R  K  E  I  I  R  N  K  I  400

CGAGCAATTGGCAAGATGGCAAGAGTCTTC TCTGTTCTCAGGGAGGAGAGTGAAAGCGTG CTGACACTCAAGGGCCTGACTCCCACAGGG ATGTTGCCTAGTGGAGTGTTGGCTGGAGGA 1320
R  A  I  G  K  M  A  R  V  F  S  V  L  R  E  E  S  E  S  V  L  T  L  K  G  L  T  P  T  G  M  L  P  S  G  V  L  A  G  G  440

CGGCAGACCTTGCAAAGTGCCACAGTTGAG GCTATTGAGGCTGAAAAAGCAATACGAGGA TTCTCTCCACCACATAGAATCTGCAGTTTT GGAGAGGCAAAGGGTTTGGATAGGATCAAT 1440
R  Q  T  L  Q  S  A  T  V  E  A  I  E  A  E  K  A  I  R  G  F  S  P  P  H  R  I  C  S  F  G  E  A  K  G  L  D  R  I  N  480

GAGAGAATGCCACCCCGGAAAGATGCTGTG CAGCAAGATGGTTTCAATTCCCTGAACACC GCACATACCACTGAGAACCACGGGACTGGC AACCATACTCCCCAGTGACCAGCGGCTTCC 1560
E  R  M  P  P  R  K  D  A  V  Q  Q  D  G  F  N  S  L  N  T  A  H  T  T  E  N  H  G  T  G  N  H  T  P  Q  *  515

CAGGGACTCTCACATCTTGGGCCCCAAATG GACAGATCACTTCAAGGAGCTGGAGGGGTT GACCAAGGTGATGATAAATGTCATAATCTC TCTGAAGAAACCATTGTGCTTTCTGAGATC 1680
CGTGCCCCCTTCCTGGATGGAGGCTTGAGG GCCCTGGGACTTGTGCCGTCTATAAGATTG GGTAATCGCTGCCACAGAGGAGAGCAGTGA GCAAGGGGCTTGGGGCAATTTCCAGTGGAG 1800
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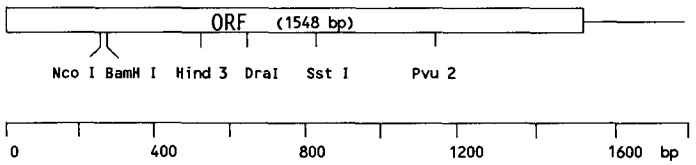

Figure 1. Nucleotide and deduced amino acid sequences for the catalytic subunit of mouse thymocyte calcineurin (type 2 gene). The nucleotide sequence of the cDNA clone, TCα-3, representing 1548 bp of open reading frame (ORF) and 252 bp of 3' untranslated region are presented. The area from bp 280 to 401, that is enclosed by a box, indicates the region used as a probe for chromosomal mapping. *Bottom of figure*, Map of unique restriction sites within the ORF.

untranslated regions, suggesting difficulty in the reverse transcription or processing of this GC-rich region during construction of the library.

Design of hybridization probes for genomic analysis

Because complex patterns of hybridization occur when large cDNA segments are used to analyze genomic blots, we examined the sequences of genes 1 and 2

TABLE 1

Comparison of the sequences of hybridization probes for the catalytic subunits of human gene 1 and gene 2

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                *       *       *  *         *  *       *
Gene 1    5' TTC TTT GAT TTG ATG AAG CTC TTT GAA GTC GGG GGA TCT CCT
Gene 2    5' TTT TTT GAT CTG ATG AAA CTT TTT GAA GTA GGA GGA TCA CCT
             F   F   D   L   M   K   L   F   E   V   G   G   S   P

              *   *   *   *       *   * * *   *   *       *
Gene 1       GCC AAC ACT CGC TAC CTC TTC TTA GGG GAC TAT GTT GAC AGA
Gene 2       GCT AAT ACA CGA TAC CTT TTT CTT GGC GAT TAT GTG GAC AGA
              A   N   T   R   Y   L   F   L   G   D   Y   V   D   R

              *   *   *       *   *       * * *       *       *
Gene 1       GGG TAC TTC AGT ATT GAA TGT GTG CTG TAT TTG TGG GC 3'
Gene 2       GGT TAT TTT AGT ATA GAG TGT GTC TTA TAT TTA TGG GT 3'
              G   Y   F   S   I   E   C   V   L   Y   L   W
                                      ═══════════════════
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The sequences shown represent those from a human brain cDNA (CNHα-3) "gene 1" and a murine thymocyte cDNA (TCα-3, this paper) "gene 2", as described under Materials and Methods. Asterisks denote differences in the nucleotide sequence of the two cDNAs and the deduced amino acid is given below each line using the one-letter code. The initial 102 bp of the sequence is contained within the third exon of the murine gene 1 coding region, whereas the region corresponding to the beginning of the fourth exon is denoted by a double underline.

for smaller regions that would make appropriate probes. Ideally, such a region should have 1) a similar exon structure for both genes 2) low homology between genes to avoid cross-hybridization and 3) high enough sequence identity between type 2 murine and human cDNAs to permit the use of a murine cDNA as a probe for human DNA. A region of 122 bp that satisfied these criteria was found in the conserved catalytic domain (Table 1, Fig. 1). Based on studies of mouse genomic clones, the majority of this region (the initial 102 bp) is contained within exon 3 of the open reading frame (S. Higuchi, R. L. Kincaid, data not shown); because the exon/intron boundary coincides exactly with the putative alternative splicing event in human gene 2 clones (13), it seems likely that this area represents a conserved exon in both genes. Although this region shows identical deduced amino acid sequence, there is only 78% nucleotide sequence identity. Finally, over this region there is only one nucleotide difference between the murine and human type 2 cDNAs. Initial experiments with probes from this region indicated that single fragments for each gene hybridized on genomic blots; this suggests that the 20 bp fragment belonging to exon 4 cannot bind under the conditions of stringency used in our experiments.

Chromosomal mapping of human genes for the catalytic subunit

Southern blot analysis of *Eco* RI-digested DNA from 27 human-hamster hybrid cell lines assigned gene 1 to human chromosome 4. A specific 9.4 kilobase (kb)

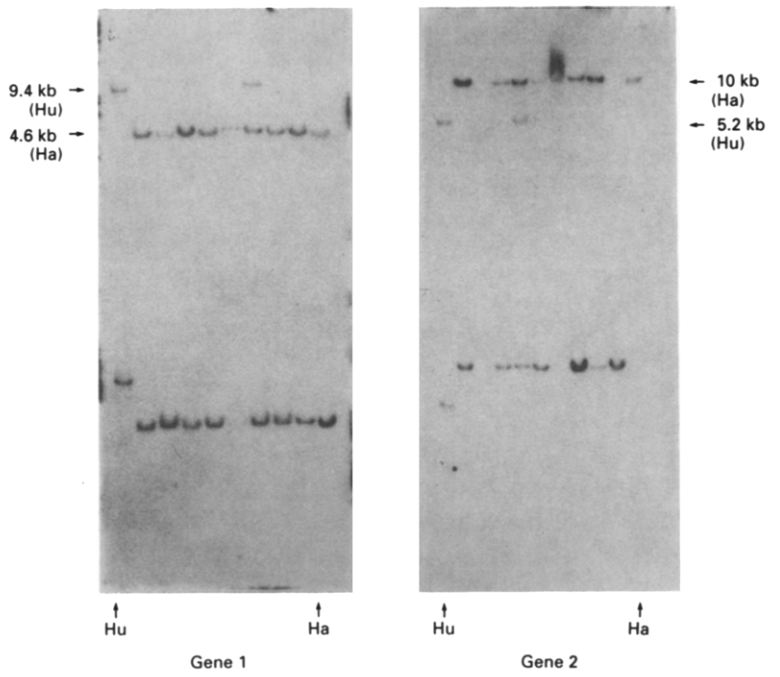

Figure 2. Assignment of calcineurin (CN) catalytic subunit genes to human chromosomes by Southern blot analysis of genomic DNA from human-hamster hybrid cell lines. Nylon membranes containing DNA from 27 different hybrid cell lines were obtained from Bios Corporation, New Haven, CT. Each lane contained 6-8 μg *Eco* RI-digested DNA. Samples were applied in two regions of the agarose gel (10 samples at the top and 10 samples in the middle) and, after electrophoresis, the DNA was transferred to GeneScreen nylon membranes (NEN). Lanes containing human (Hu) and hamster (Ha) DNA were also included as controls at the either end of the blots (see arrows). A total of three blots were used in the analysis, two of which are shown.

Left panel: Blot # 656 was hybridized to ^{32}P-radiolabeled cDNA probe for gene 1 (see table 1). Hybridization in control lanes showed a 9.4 kb fragment that is specific for human (Hu) (left side) and a 4.6 kb fragment for the hamster (Ha) homologue (right side).

Right panel: blot # 801 was hybridized with ^{32}P-radiolabeled gene 2 probe (see table 1). Hybridization in control samples showed a 5.2 kb fragment that is specific for human (Hu) (left side) and a 10 kb fragment for the hamster (Ha) homologue (right side).

fragment, corresponding to the control sample of human DNA (lane 1), was present in two hybrid cell lines containing chromosome 4 (e.g. lane 7 upper panel; Fig. 2), and was absent in all hybrids lacking this chromosome. Assignment of the gene 2 locus was made by analysis of the same 27 human-hamster hybrid cell lines. A 5.2 kb human-specific band (lane 1) segregated only with human chromosome 10 (e.g. lane 5, upper panel; Fig. 2). The validity of these assignments was confirmed using markers that map to a particular chromosome. Probes for α-fetoprotein (present on chromosome 4), and the ß-subunit of F1-ATPase, (chromosome 10) reacted only with the cell lines recognized by the gene

1 and gene 2 probes, respectively (data not shown). Thus, the data obtained using somatic cell hybrids demonstrate clearly that gene 1 and gene 2 catalytic subunits reside on different human chromosomes and suggest that their expression is not likely to be linked physically. Because the exact localization of these genes is not yet known, studies using *in situ* hybridization and pulse field-electrophoresis will be needed to identify their precise chromosomal sites.

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