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Calcineurin A alpha (PPP3CA), calcineurin A beta (PPP3CB) and calcineurin B (PPP3R1) are located on human chromosomes 4, 10q21→q22 and 2p16→p15 respectively

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Abstract. Calcineurin (also called protein phosphatase-2B) is a calmodulin-regulated protein phosphatase which plays an important role in signal transduction. The enzyme is a heterodimer of a 58–59 kDa calmodulin-binding catalytic subunit (calcineurin A) and a small (i.e. 19 kDa) Ca^{2+}-binding regulatory subunit (calcineurin B). The highly conserved calcineurin B is encoded by a single gene in all tissues except testes, whereas there are three isoforms of calcineurin A (α, β and γ) encoded by genes on three different chromosomes. This enzyme can play a critical role in transcriptional regulation and growth control in T lymphocytes by a mechanism believed to involve dephosphorylation of the nuclear factor NF-AT which is essential for transcription of the interleukin-2 gene. To better evaluate the potential role of the calcineurin genes in human genetic disorders, we have studied their chromosome locations. Calcineurin B (PPP3R1) is located on human chromosome 2p16→p15 and calcineurin Aβ (PPP3CB, previous gene symbol CALNB) is present on 10q21→q22. We confirm the localization of calcineurin Aα (PPP3CA, previous gene symbol CALNA) to chromosome 4 without regional localization.

Calcineurin, a calmodulin-regulated protein phosphatase, is found in the cells of all eukaryotes ranging from yeast (Cyert and Thorner, 1989) to mammals (reviewed by Klee et al., 1987; Guerini and Klee, 1991; Kincaid et al., 1991). This heterodimeric protein consists of a 19 kDa Ca^{2+}-binding regulatory subunit, calcineurin B, and a 58–59 kDa catalytic subunit, calcineurin A (Klee et al., 1987). One gene encodes calcineurin B in all tissues except testes (Mukai et al., 1991), and it is highly conserved at the level of both protein and DNA sequences in eukaryotes (Guerini and Klee, 1989). The testes-specific form of calcineurin B has also been cloned (Sugimoto et al., 1991; Mukai et al., 1991; Ueki et al., 1992). In contrast, there are two major isoforms (Aα, Aβ) of calcineurin A encoded by separate genes located on different human chromosomes (Giri et al., 1991) and on chromosome 15 in the rat (Yamada et al., 1994). A third isoform (Aγ) is unique to testes (Tash et al., 1988; Muramatsu and Kincaid, 1992). Additional diversity of calcineurin A is created by alternative splicing of mRNAs (Guerini and Klee, 1989; Giri et al., 1991; McPartlin et al., 1991).

Although calcineurin is especially abundant in brain where it constitutes 1% of the total protein, it is found at lower levels in all mammalian tissues examined (Klee et al., 1987). Interestingly, abundant amounts of calcineurin B mRNA have also been found in some tumor (i.e. HeLa) cells (Guerini et al., 1989), although the protein product itself was not abundant in these cells.

Regulation of calcineurin by Ca^{2+} implies that this enzyme plays an important role in signal transduction and recent studies have disclosed the role of calcineurin in T lymphocyte activation (Liu et al., 1991 and reviewed in Schreiber et al., 1992; Clipstone and Crabtree, 1993). Thus, activation of NF-AT, the nuclear factor of activated T cells which is essential for transcription of the interleukin-2 gene upon T cell activation, requires dephosphorylation of NF-AT by calcineurin and its

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translocation from cytosol to nucleus (McCaffrey et al., 1993). Of interest is the fact that the immunosuppressive drugs cyclosporin A and FK506 both function by inhibiting calcineurin through the formation of a complex between the drug, its specific binding protein, and the phosphatase. Considering the importance of calcineurin in regulation of transcription and growth, we have determined the chromosome locations of the genes for calcineurin A and B with the expectation that this information might provide clues to possible roles of these genes in neoplastic genetic or other hereditary diseases.

Materials and methods

Somatic cell hybrids

Human and rodent parental cells, cell fusion technique, and isolation and characterization of hybrids have been described elsewhere (McBride et al., 1982). Hybrid cells were analyzed for the presence of all human chromosomes except Y by standard isoenzyme analyses, as well as by Southern analysis with probes from previously localized genes and, frequently, by cytogenetic analysis. Southern blots of DNA restriction digests of hybrid cells on positively charged nylon membranes were prepared after (0.7%) agarose gel electrophoresis and hybridized at high stringency with ^{32}P-labeled cDNA probes (Gnarra et al., 1990). Genes were mapped to specific chromosomes by correlating cell lines retaining the hybridizing human sequences with the specific human chromosomes retained in each of the somatic cell hybrids.

Chromosomal in situ hybridization

In situ hybridization experiments were performed as described previously (Gnarra et al., 1990). Chromosomes were prepared from peripheral blood lymphocytes from a normal male (46;XY) cultured for 72 h at 37°C in RPMI 1640 supplemented with 15% fetal bovine serum, phytohemagglutinin (0.5 µg/ml), and antibiotics. Cultures were synchronized by addition of BrdU (100 µg/ml) for 16 h. Chromosomal DNA on slides was denatured for 3 min in 0.07 N NaOH in 64% ethanol (Singh et al., 1977; Landegent et al., 1985). Radiolabeled probes (specific activity 3×10^7 cpm/µg) were prepared by nick translation of plasmid DNA with ^{3}H-TTP and ^{3}H-dCTP. Hybridization in a solution containing 50% formamide and 5% dextran sulfate was carried out for 20 h at 42°C, and slides were washed in 50% formamide, 2 × SSC (pH 7.0) at 42°C. The slides were coated with NTB2 nuclear track emulsion, stored desiccated at 4°C, developed, stained (0.25% Wright stain), and photographed. After destaining, chromosome banding was obtained by staining with Hoechst 33258 (150 µg/ml) for 30 min and exposing to UV light for 30 min after rinsing. The slides were again stained with Wright stain and the same metaphase spreads were rephotographed (Bhatt et al., 1988).

Preparation of probes

The cDNA sequence (2558 bp), deduced amino acid sequence, and restriction map of human calcineurin B (PPP3R1) has been reported (Guerini et al., 1989). Three DNA subfragments were isolated from this cDNA by agarose gel electrophoresis and electroelution after digestion with *Eco*RI (Fig. 1A). A 1068-bp fragment (B3) contained 60 bp of 3′-coding sequence and 1008 bp 3′-UTR; a 234-bp fragment (B2) contained the middle coding region; and a 300-bp fragment (B1) contained about 80 bp 5′-UTR and the 5′ portion of coding sequence extending to an *Eco*RI site at Glu73. The complete nucleotide and deduced amino acid sequence of human calcineurin Aβ (PPP3CB) has also been reported; clones containing two types of cDNA were identified which differed only in their 3′ coding and 3′ untranslated regions (Fig. 1B) presumably due to alternative splicing (Guerini and Klee, 1989). Contiguous subfragments of these cDNAs were isolated and used as probes (Fig. 1B). Probe A1 is a 560-bp internal coding fragment isolated from a 5′ truncated cDNA which extends to a *Sac*I site at nucleotide 863 of calcineurin Aβ2. Probe A2 was a 330-bp *Sac*I to *Pst*I fragment. Probe A3 was a 235-bp *Pst*I-*Pst*I fragment containing 169 bp common to both calcineurin Aβ1 and calcineurin Aβ2 cDNAs as well as 66 bp unique to calcineurin Aβ2 cDNA. Probe A4 was unique to calcineurin Aβ2 cDNA extending from a *Pst*I site through 145 bp of 3′ coding sequence and extending about 1500 bp to the 3′ end of the cDNA. Probe A5 was an 850-bp 3′ fragment of calcineurin Aβ1 cDNA extending from the *Eco*RI site 87 bp beyond the point of divergence of calcineurin Aβ1 and calcineurin Aβ2 to the 3′ end of the cDNA. Using the sequence for human calcineurin Aα determined by Kincaid et al. (1990), a 503-bp fragment (nts 1491–1993) containing the 3′ coding and 3′ untranslated region of this gene was prepared by PCR amplification. Human genomic DNA was used as template, and a 687-bp fragment was first isolated by gel electrophoresis in low melt agarose after PCR amplification using oligonucleotide primers 5′-CCGAATTAATGAGAGGATGCCG-3′ (nucleotides 1431–1452) and 5′-GACTGCCTAATTCAGTTTATAGCC-3′ (nucleotides 2117– 2094). This denatured DNA fragment was then used as template for PCR amplification with the nested primers 5′-CTCCATCAACAAGGCTCTCACC-3′ (nucleotides 1491–1512) and 5′-TAGTGCTGCGACTGTAAACGTAC-3′ (nucleotides 1993–1971). DNA fragments were labeled with ^{32}P-dCTP by random hexanucleotide primed DNA synthesis (Feinberg and Vogelstein, 1983) to specific activity > 10^9 cpm/µg DNA prior to use as probes.

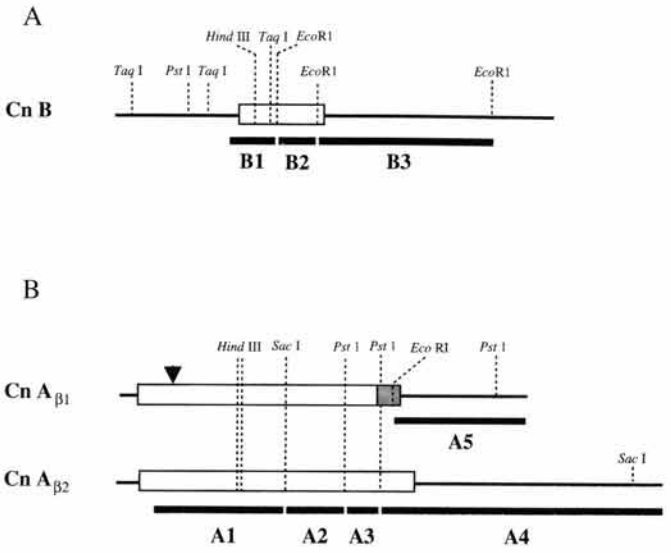

Fig. 1. Hybridization probes used to chromosomally map calcineurin genes. (**A**) Partial restriction map of PPP3R1 cDNA and subfragments used as probes. All three insert fragments were pooled and used as a probe for in situ hybridization (Fig. 2). (**B**) Restriction map of calcineurin Aβ1 and calcineurin Aβ2 cDNA clones and subfragments used as probes. Coding sequences are indicated in boxes. Sequences of calcineurin Aβ1 and calcineurin Aβ2 genes downstream from the 5′ end of the stippled region are completely divergent. The arrow indicates the position of a 54 bp insert in calcineurin Aβ1 cDNA. The 850-bp 3′ fragment of calcineurin Aβ1 cDNA (probe A5) and a 2-kb fragment of calcineurin Aβ2 cDNA (probes A2, A3, and A4 combined) were each labeled with ^{3}H and the mixture was used for in situ hybridization.

Results

Localization of PPP3R1 on chromosome 2

A panel of human/rodent somatic cell hybrids segregating human chromosomes was used to map calcineurin genes to specific human chromosomes by Southern hybridization of the DNAs with probes for each of the genes. PPP3R1 was localized to human chromosome 2 by Southern hybridization of the somatic cell hybrid DNAs with B3, a 1068-bp 3′ cDNA probe. A 2.4-kb human hybridizing band was well resolved from the 8.7-kb crosshybridizing Chinese hamster band (Fig. 2A) and

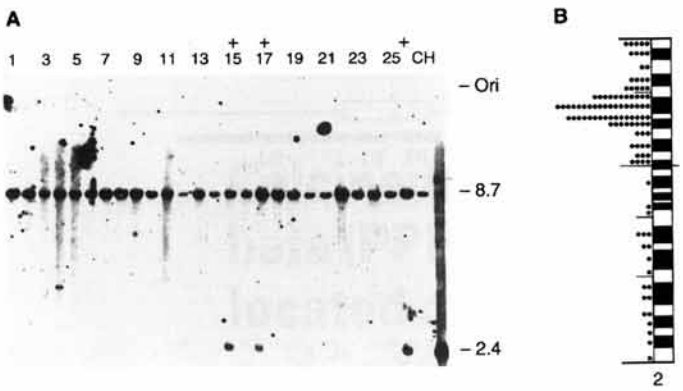

Fig. 2. (**A**) Southern hybridization of representative human/hamster somatic cell hybrid *Eco*RI DNA digests with PPP3R1 probe B3. A different hybrid cell DNA is present in each of the numbered lanes, and parental Chinese hamster (C) and human placental (H) DNAs are also shown. Lanes 16 and 17 contain DNA from hybrids containing human t(2;6)(q11;q15) translocation chromosomes; the 2pter→q11 translocation chromosome is present in lane 17 (PPP3R1 present) whereas the 2q11→qter translocation chromosome is present in lane 16 (PPP3R1 absent). An intact chromosome 2 is present in lane 15 and 2pter→cen fragment is present in lane 26 (spontaneous chromosome break); human PPP3R1 is retained in both of these hybrids. (**B**) Ideogram showing the distribution of grains on chromosome 2 after hybridization with PPP3R1.

the results were confirmed by hybridization with probes B1 and B2. The gene segregated concordantly with human chromosome 2 and discordantly (≥20%) with all other chromosomes (Table I). The gene was further localized to the short arm of chromosome 2 by examination of hybrids containing well-characterized translocations and spontaneous breaks. Two human/hamster hybrids isolated after fusion of human fibroblasts (GM2658) containing a t(2;6)(q11;q15) reciprocal translocation (McBride et al., 1983) retained only one of the two translocation chromosomes in the absence of a normal chromosome 2 (Fig. 2). The hybrid retaining the 2pter→q11 translocation (lane 17) retained human PPP3R1 whereas the hybrid retaining the reciprocal translocation chromosome (lane 16) did not. Another human/hamster hybrid contained a spontaneously broken human chromosome 2 which had lost the entire long arm of this chromosome but retained human PPP3R1 (lane 26). A human/mouse hybrid containing human PPP3R1 retained only the human X chromosome and a fragment of human chromosome 2p; the break in chromosome 2 was distal to the immunoglobulin kappa locus (2p12) and allowed localization of PPP3R1 to 2pter→p12 (not shown).

In situ hybridization with a ^{3}H-labeled PPP3R1 cDNA probe allowed more precise localization of the gene. A total of 103 metaphases containing a grain on chromosome 2 were analyzed before and after G banding. There were 204 total grains (2.0 grains/spread) and 107 grains (52% of total) were located on chromosome 2 with 87 (81%) of these grains on the short arm. A peak of 34 grains was centered over 2p16→p15 (Fig. 2B) and an additional 24 grains were located over the two adjacent bands (2p14 and 2p21).

Table I. Mapping of calcineurin B, Aα and Aβ genes to chromosomes 2, 4 and 10 by Southern hybridization of human cDNA probes with *Eco*RI digests of human/rodent somatic cell hybrid DNA

Human chromosome	% Discordancy		
	PPP3R1[a]	PPP3CA[b]	PPP3CB[c]
1	20	21	34
2	0	25	29
3	35	20	38
4	42	0	51
5	21	28	28
6	42	38	36
7	37	53	40
8	32	33	21
9	23	28	35
10	26	33	0
11	22	28	30
12	33	29	26
13	28	45	32
14	38	54	41
15	40	54	53
16	44	44	32
17	41	36	55
18	42	39	49
19	25	35	33
20	32	27	38
21	65	47	60
22	32	36	31
X	53	39	45

Discordancy represents presence of the gene in the absence of the chromosome or absence of the gene despite the presence of the chromosome The percent discordancy is the sum of these numbers divided by total hybrids examined (× 100).

[a] The cDNA probes are shown in Fig. 1A. The human/hamster hybrids consisted of 27 primary clones and 14 subclones (5 positive of 41 total) and the human/mouse hybrids consisted of 14 primary clones and 40 subclones (17 positive of 54 total).

[b] The cDNA probe was a 506-bp fragment prepared by PCR amplification as indicated in Methods. The human /hamster hybrids consisted of 26 primary hybrids and 13 subclones (15 positive of 39 total) and human/mouse hybrids consisted of 18 primary and 28 subclones (26 positive of 46 total)

[c] The cDNA probes are shown in Fig. 1B. The human/hamster hybrids consisted of 29 primary clones and 14 subclones (19 positive of 43 total) and the human/mouse hybrids consisted of 18 primary clones and 35 subclones (3 positive of 53 total).

Localization of PPP3CB to human chromosome 10

PPP3CB was mapped to human chromosome 10 (Table I) by Southern analysis of the same panel of human/rodent somatic cell hybrids with PPP3CB probes, and the gene segregated discordantly (≥21%) with all other human chromosomes. Hybridization was observed with each of the probes for calcineurin Aβ in the same hybrid cell lines. All hybridizing bands could be explained by the presence of a single gene, and probes specific to calcineurin Aβ1 or calcineurin Aβ2 cDNAs identified hybridizing bands in the same hybrid cell lines.

In situ hybridization was then performed using a mixture of probes for both calcineurin Aβ1 and calcineurin Aβ2 cDNAs. There were a total of 122 grains and 61 (50% of total) were located on chromosome 10. The random distribution of grains on chromosome 7 (6 grains), 8 (23 grains), 9 (17 grains) and 11 (15 grains) reflected nonspecific background hybridization. In contrast a peak of 26 grains was centered on band 10q21 and 10 additional grains were on the adjacent band 10q22 (Fig. 3). The gene was localized to the proximal long arm (10q21→q22).

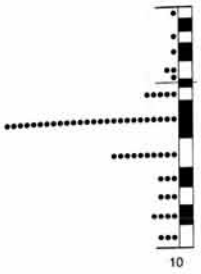

Fig. 3. Ideogram showing the distribution of grains on chromosome 10 hybridized with PPP3CB probe.

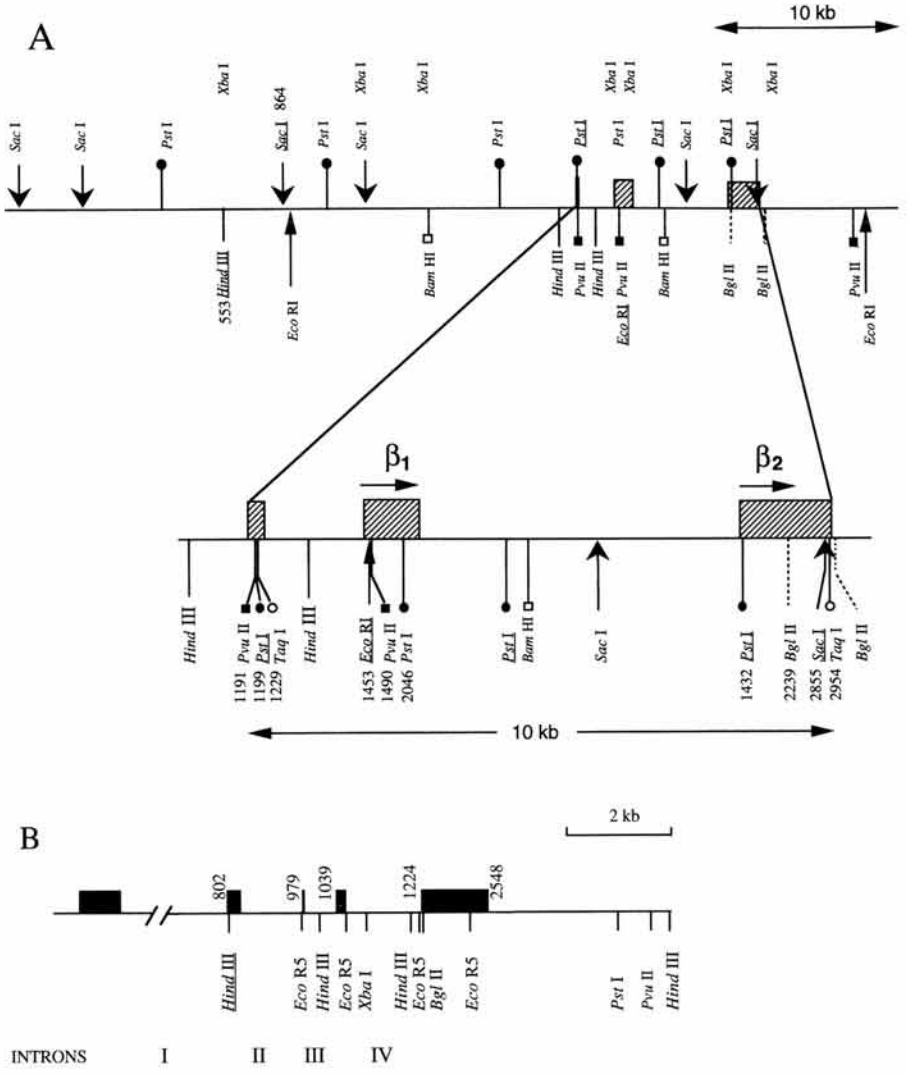

Fig. 4. Genomic organization of calcineurin A genes. (**A**) The 3′ portion of the human calcineurin Aβ gene cluster as determined by Southern hybridization of blots of restriction digests of human DNAs with various PPP3CB cDNA probes. Sequences present in the cDNAs are shown as stippled bars. Restriction sites also shown in Fig. 1A and 1B are underlined. (**B**) Organization of PPP3R1 including sequences from human chromosome 20 (nt 1– <nt 547) fused to the 5′ end of the putative 5′-UTR. Exons 1–5 are shown as solid bars. The numbers correspond to nucleotides in the cDNA sequences of calcineurin A and calcineurin B genes (Guerini and Klee, 1989; Guerini et al., 1989).

Mapping of PPP3CA to human chromosome 4

A 503-bp fragment prepared by PCR amplification of human genomic DNA was used as a probe, and it consisted predominantly of the 3′ untranslated region of the α isoform gene. This probe identified a 2.6-kb hybridizing human band in *Eco*RI digests which was adequately resolved from 2.9-kb cross-hybridizing bands in mouse and Chinese hamster DNAs, and there was no cross-hybridization with the gene for the β isoform of calcineurin A. Analysis of the hybrid panel allowed confirmation of the assignment of this gene to human chromosome 4, and the gene segregated discordantly (≥20%) with all other human chromosomes.

Examination for RFLPs at calcineurin loci

All probes were hybridized with blots containing restriction digests (*Eco*RI, *Hin*dIII, *Bam*HI, *Xba*I, *Sac*I, *Taq*I, *Msp*I, *Bgl*II, *Pvu*II, *Pst*I, *Eco*RV, and *Kpn*I) of DNAs isolated from 10 unrelated individuals. No restriction fragment length polymorphisms were detected. These same blots were used for determination of the genomic organization of PPP3CB and demonstration that calcineurin Aβ1 and calcineurin Aβ2 are created by alternative splicing.

Two types (i.e. 1 and 2) of cDNA clones for calcineurin Aβ were previously isolated by Guerini and Klee (1989), and they were thought to represent alternative splicing events. This conclusion was based upon the fact that the cDNAs appeared to be identical throughout their 5′ regions, whereas the sequences diverged completely starting at a point in the 3′ coding region. This interpretation has now been confirmed by Southern analysis of human genomic DNA restriction digests with the calcineurin Aβ probes, and the organization of the 3′ portion of the gene has been determined (Fig. 4A). Hybridization of the same fragment with multiple probes could be distinguished from hybridizing bands of closely similar sizes by reusing the same blots for hybridization with each of the probes after removing the previous probe from a blot by treatment with alkali. Only a 13.3-kb *Eco*RI band was identified by both probes A4 (type 2 specific) and A5 (type 1 specific); probe A3 hybridized strongly with a 17.6-kb band and weakly with the same 13.3-kb band (due to the 66 bp 3′ portion of this probe), and probe A2 hybrid-

ized with the 17.6-kb band as well as 1.2- and 3.2-kb bands. Only a 14.5-kb *Bam*HI fragment hybridized with probes A3 and A5 whereas only a 28-kb band was identified with probe A4. Probes A3, A4, and A5 all hybridized with the same 9.5-kb fragment in *Taq*I digests, and there were no other hybridizing bands. In *Sac*I digests, probes A2, A3, and A5 (but not A4) all identified the same 17.4-kb band, and A2 also hybridized with a 4.6-kb band; probe A4 hybridized only with a 3.9-kb band which also was identified by probe A3 (due to the 66 bp 3′ end of the probe). In *Pst*I digests, a 2.6 kb-band was detected with probes A3 and A5; bands of 4.1 and 1.9 kb were also detected with probes A3 and A5, respectively, and a 3.7 kb band was identified by probe A4. Results of analysis of digests with the other restriction endonucleases supported and extended these analyses and permitted construction of a map of this portion of PPP3CB (Fig. 4A). Calcineurin Aβ1 and calcineurin Aβ2 cDNAs diverge at the position of a 1.8-kb intron (2.6-kb genomic fragment minus 0.8-kb cDNA spanned in this region). Calcineurin Aβ2 cDNA is generated by alternative splicing at this same site which involves loss of the exon containing the 3′ end of calcineurin Aβ1 cDNA as well as the next intron; the distance spanned by these two introns and exon are estimated to be about 8 kb as determined by the difference in distance between the most 3′ *Taq*I sites in genomic DNA (9.5 kb) and the same distance in the cDNA (1.7 kb). The portion of the calcineurin A gene containing only probes A2, A3, A4, and A5 represents about 26 kb, indicating that the entire gene spans a relatively large distance.

Genomic organization of PPP3R1

Genomic cloning, PCR amplification, and DNA sequencing studies were used to determine the rough genomic organization of calcineurin B (Fig. 4B). Three clones were used for these purposes including a genomic 5-kb *Hin*dIII fragment which contains 147 bp at the 3′ end of intron IV and extends to a *Hin*dIII site in the 3′ flank of the gene. Another subclone designated HUBS 11 contained only exon 2 and about 500-bp of contiguous sequence from intron I and about 200 bp from intron II. Intron I could not be PCR amplified using as templates various primers derived from the 5′ cDNA clone Hg2 (Guerini et al., 1989), genomic clones or high molecular weight human DNA. A cloning artifact had been suggested to explain the failure to demonstrate by S1 mapping that the 5′ end of clone Hg2 hybridizes with Hela cell mRNA (Guerini et al., 1989). This conclusion was confirmed by the demonstration of the chimeric nature of the 5′-UTR of the Hg2 clone. A 195-bp fragment (nts 5–199) of Hg2 was used as a probe for the hybrid DNA panel and this sequence clearly mapped to human chromosome 20. A 239-bp probe of Hg2 (nts 309–547) detected two human bands in *Bam*HI digests of hybrid DNAs; a 3.2-kb band is located on chromosome 20 whereas a 5.4-kb band is present on human chromosome 2. Primarily by PCR amplification of genomic segments, it could be demonstrated that the calcineurin B gene contains four introns (Fig. 4B) with sizes of >4.6, 1.1, 0.6, and 1.4 kb. PCR generated products were also purified and used as probes for Southern hybridization analyses.

Discussion

The importance of the Ca^{2+} and calmodulin-dependent serine/threonine phosphatase, calcineurin, is suggested by its presence in all eukaryotes. A vital role for this enzyme has been described in T-lymphocytes (Liu et al., 1991). Binding to DNA by NF-AT, a regulatory protein, is essential for transcription of the interleukin-2 gene upon T cell activation. Dephosphorylation of NF-AT by calcineurin is required for translocation of NF-AT from cytosol to nucleus and its subsequent specific binding to DNA (McCaffrey et al., 1993). The immunosuppressants, cyclosporin A and FK506, act by inhibition of calcineurin through non-competitive binding to the enzyme of the drugs bound to their specific receptor proteins (immunophilins) (Liu et al., 1991). Calcineurin phosphatase activity is also a critical mediator of T cell receptor/CD3 signaling leading to programmed cell death (apoptosis) in T cell hybridomas (Fruman et al., 1992). This enzyme is also involved in the α-adrenergic stimulation of the Na^+,K^+-dependent ATPase in kidney (Aperia et al., 1992), the release of glutamate from nerve terminals (Nichols et al., 1994), the nerve terminal depolarization (Liu et al., 1994), long term depression (Mulkey et al., 1994), the regulation of the K^+ channels in plant cells (Luan et al., 1993) and the response to mating factor in yeast (Cyert and Thorner, 1992). Calcineurin is present in especially high levels in brain where it is thought to be involved in the action of several neurotransmitters or neuromodulators (Goto et al., 1992).

The evolutionary conservation of multiple isoforms of the catalytic subunit, calcineurin A, is striking. In addition to testes specific forms of both calcineurin A and calcineurin B, all mammals contain two isoforms of calcineurin A which each interact with a common regulatory subunit. One major difference in the two isoforms is the presence of a polyproline tract (i.e. eleven residues) near the N-terminus of calcineurin Aβ. Evolutionary conservation of these two isoforms probably allows tissue specific regulation of expression of this phosphatase but the precise distribution of each isoform in tissues has not yet been determined. At least two isoforms of calcineurin A are found in Drosophila (Guerini et al., 1992; Brown et al., 1994) and yeast (Cyert et al., 1992).

PPP3CB is a relatively large gene with many introns, and it spans at least 50 kb. In contrast, the PPP3R1 probably contains only four introns and it apparently spans about 12 kb. The published sequence (Guerini et al., 1989) had to be revised with respect to the 5′-UTR. It appears that the putative 5′-UTR is a chimeric sequence representing a fusion product of some sequence on chromosome 20 with PPP3R1 on chromosome 2p16→p15. Of interest is the fact that the calcineurin B gene in Drosophila (Guerini et al., 1992) contains no introns while the calcineurin B gene in yeast contains a single intron at the same position as intron I in the human gene (Cyert and Thorner, 1992).

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