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Cloning and Differential Expression of New Calcium, Calmodulin-Dependent Protein Kinase II Isoforms in *Xenopus laevis* Oocytes and Several Adult Tissues[1]

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The different oligomers composing the high molecular weight calcium/calmodulin-dependent protein kinase II (CaMKII) holoenzyme, previously shown to be transiently activated during *Xenopus* oocyte maturation, migrate on SDS-PAGE as proteins of 83, 72, 62, 56, and 52 kDa and have all been cloned. The holoenzyme consists of the CaMKII isoforms γB, γC, and δ12, already described in other species, while γJ, γK, γL, γM, and γN are now described for the first time. The γ-isoforms are splice variants of the γ-gene, containing in their variable region different combinations of known exons and one, two or three novel exons. Semi-quantitative RT-PCR revealed that all isoforms identified in prophase oocytes are also expressed in adult tissues with a tissue-specific expression pattern. At least thirty different CaMKII isoforms could be identified in different *Xenopus* adult tissues, most of which are described here for the first time.

Key words: calcium/calmodulin-dependent protein kinase II, CDK1, isoforms, maturation, *Xenopus laevis*.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous, multifunctional serine/threonine kinase involved in various cellular functions regulated by calcium (*1*). It can be distinguished from other calcium/calmodulin-dependent protein kinases through its structural, regulatory and enzymological properties. The 300–700 kDa kinase is a multimeric protein composed of 6–12 individual protein kinase subunits (reviewed in Refs. *2* and *3*). The subunits are the products of four separate genes, α, β, γ, δ, which are expressed in a tissue-specific manner. Two of the isoforms (α and β) are restricted largely to neuronal tissues, whereas the γ- and δ-isoforms have a more widespread tissue distribution (*4*). Additional subunit diversity arises through alternative splicing. All isozymes contain a catalytic, a regulatory, a variable and an association region. Most differences between the 4 CaMKII genes and among the splice variants are located in their variable region (*3*).

Previous studies have assigned a role to CaMKII in the regulation of the cell cycle and, more specifically, during entry and exit from meiosis (*5*). *Xenopus laevis* oocytes are widely used to study these transitions. Oocytes taken from the ovary of the frog are arrested in prophase of the first meiotic division. The addition of progesterone results in the activation of CDK1, causing meiotic progression and a second arrest in metaphase of the next meiotic division. At this stage, oocytes are also called eggs and are ready to be fertilized. The whole process is called oocyte maturation (reviewed in Ref. *6*). As CaMKII is a calcium/calmodulin-dependent protein kinase, the study of its activation was intimately linked to the study of calcium transients throughout the cell cycle. Whereas a clear calcium transient and corresponding function of CaMKII was shown at fertilization (exit of meiosis) (*5*), evidence for a calcium transient and CaMKII activation during oocyte maturation (meiosis entry) was less equivocal. However, Han and Lee (*7*) showed that microinjection of the calcium-chelator BAPTA or a monoclonal antibody against phosphatidylinositol 4,5-biphosphate (PIP2) inhibits maturation, and that microinjection of a constitutive active mutant of CaMKII can initiate, but not complete, maturation (no real GVBD occurred) (*8*).

Recently, we provided a new impetus to the idea of the involvement of CaMKII in the activation of oocyte matura-

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Abbreviations: AIP, autocamtide-2–related inhibitory peptide; BAPTA, 1.2-bis(*o*-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; CaMKII, calcium/calmodulin-dependent protein kinase II; CDK1, cyclin-dependent kinase 1; GVBD, germinal vesicle breakdown; RT-PCR, reverse transcriptase–polymerase chain reaction.

tion starting from a different angle. By studying the kinase(s) responsible for the phosphorylation of cyclin B2 during maturation, we identified a cell cycle–regulated kinase that specifically phosphorylated cyclin B2 on serine 53, and named it Cyk (*9*). Subsequently we purified Cyk to homogeneity as a holoenzyme of about 670 kDa and could identify it as a novel form of CaMKII (*10*). Cyk/CaMKII consists of five isoforms with molecular masses ranging from 52 to 83 kDa, all autophosphorylating and reacting with CaMKII antibodies and with calmodulin in an overlay experiment. Tryptic peptide sequencing combined with the size of the isoforms revealed that at least three of them could be considered novel isoforms. Moreover, through direct *in vivo* measurement, we were able to demonstrate a transient activation of Cyk/CaMKII during *Xenopus* oocyte maturation, and through microinjection of the calcium-chelator BAPTA or the specific CaMKII inhibitor AIP we established the importance of Cyk/CaMKII in the maturation process. These findings prompted us to characterize Cyk/CaMKII at the molecular level.

We now report the cloning of eight different CaMKII isoforms, covering all isoforms present in the holoenzyme as isolated from prophase oocytes. In addition, the isoform distribution of these and other β-, δ-, γ-isoforms were investigated in several adult tissues using a semi-quantitative RT-PCR strategy, and at least thirty different CaMKII isoforms were found in *Xenopus*, most of them described for the first time. This knowledge will provide the structural basis for further functional analysis.

EXPERIMENTAL PROCEDURES

PCR Analysis—A λgt10 *Xenopus* oocyte cDNA library (kindly provided by Dr. D.A. Melton, Massachusetts, USA) was subjected to nested PCR, essentially as described (*11*). The primers used were derived from the cDNA of the *Xenopus* CaMKII β isoform (genbank database, accession number U06636). For the primary PCR reaction, the sense primer 5′GT**TCTAGA**TGGGACACGGTGACCCCTGAAGCC3′ (encoding the amino acid sequence WDTVTPEA from the catalytic domain; the *Xba*I restriction site is shown in bold) and the antisense primer 5′GTT**GGATCC**AGGATTTAGGATGGTGGTGTG3′ (encoding HTTILNP from the association domain; the *Bam*HI restriction site is shown in bold) were used. For the secondary (nested) PCR reaction we used 5′T**TCTAGA**GGGGCCATCCTCACAACAATGCTG3′ (encoding GAILTTML) as the sense primer (*Xba*I restriction site in bold) and 5′GTT**GGATCC**CAGGTTACCAAGAGCTTCCGG3′ (encoding PEALGNL) (*Bam*HI site in bold) as the antisense primer, both flanking the variable region borders. PCR reactions were performed in 50 μl using 1 U PWO DNA polymerase (Boehringer), PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol primer, and 1 μl of a cDNA library consisting of 20 million plaque phage units. A five minutes denaturation step at 96°C was followed by 30 cycles of eighty seconds denaturation at 96°C, 2 min annealing at 55°C, and 3 min elongation at 72°C. The PCR products generated were cloned into the pBluescript II SK^- vector. The CaMKII variable regions obtained were sequenced.

cDNA Library Screening and Sequencing—A specific probe for the high molecular weight protein bands of CaMKII purified from prophase *Xenopus* oocytes (*10*) was obtained by performing PCR, essentially as described above. Therefore, one of the sequenced variable regions was used as a template with 5′TTTCTAGAGGGGCCATCCTCACAACAATGCTG3′ (encoding GAILTTML) as the sense primer and 5′GACACCATCTGTCTTCTTGTTC3′ (encoding LNKKTDG) as the antisense primer. To cover the whole range of CaMKII isoforms, a second probe was made by performing PCR using the cDNA of the *Xenopus* β isoform (kindly provided by T.R. Soderling, Portland) as template with 5′GTTCTAGATGGGACACGGTGACCCCTGAAGCC3′ (encoding WDTVTPEA) as the sense primer and 5′GCCAGCATTGTCGTGAGGATGGCCCC3′ (encoding GAILTTML) as the antisense primer. Probes were labeled with a random priming DNA labeling kit (Roche). The *Xenopus* oocyte λgt10 library was screened, positive clones were isolated, and the plaques purified, as described previously (*12*). Isolated pure clones were digested with *Eco*RI and cloned into the pBluescript II SK^- vector. Nucleotide sequences were determined with a Thermo sequenase fluorescent labeled primer cycle sequencing kit and an A.L.F. Express sequenator (Pharmacia LKB) using T7, T3, the forward primer 5′GAACACCTAACATTGTGCGTCTCC3′ and the reverse primer 5′AAAATTTATGGAAGTCCATCCCTTC3′. In addition to the original clones, we used subclones obtained by restriction enzyme digestion as templates for DNA sequencing.

In Vitro Transcription and Translation—[^{35}S]Methionine-labeled translation products were obtained using the TNT Coupled reticulocyte lysate system (Promega) with the cDNAs cloned into the pBluescript SK^- vector as template and the appropriate RNA polymerase (T3 and T7). The translation products were subjected to SDS-PAGE (12%) and visualized by autoradiography.

Purification of CaMKII from Prophase Xenopus Oocytes—CaMKII was purified from prophase *Xenopus* oocytes as described (*10*).

Semi-Quantitative RT-PCR and Southern Blotting—Prophase oocytes were prepared as described (*13*). Several *Xenopus* adult tissues (liver, muscle, heart, spleen, gall bladder, adipocytes, brain) were excised from adult female frogs and immediately freeze clamped in liquid nitrogen. Total RNA from prophase oocytes and adult tissues were prepared as described (*14*). RNA was further purified using a mini Qiagen RNA purification kit (Westburg). cDNA was prepared from 2 μg of total RNA using the "stop"-primer (5′GGAATTCGGATCCTCACTGGAGAGGGGCTGCTGG3′) for γ-isoforms or the "stop"-primer (5′GGGAATTCCTTAATTCGGTATTGTTGGTGAGCC3′) for δ-isoforms having the conventional carboxyl terminus (no C-terminal extension) or the "stop"-primer (5′GGAATTCCCATCACTGGAGGGGTGCAACGG3′) for β-isoforms and 20 U M-MuLV-reverse transcriptase (Fermentas). Subsequently, the cDNA was purified using a GFX DNA-purification kit (Pharmacia) and PCR was performed essentially as described above. However, to be in the linear DNA amplification range, only 20 amplification cycles were performed with the primers 5′CACAGGCAAGAAACCGTGGAATGCTTGAG3′ and 5′GTGTAGGCTTCAAAATCCCC3′ (encoding RQETVECLR and GDFEAY, respectively) for γ-isoforms, the primers 5′GCAAGAAGAAAACTAAAGGGGG3′ and 5′ACTAGATTTCCCAGAGCTTCTGG3′ (encoding for ARRKLKG and PEALGNL, respectively) for δ-isoforms, and the primers 5′GTTCTAGATGGGACACGGTGACCCCTGAAGCC3′ and 5′G-

TTGGATCCCAGGTTACCAAGAGCTTCCGG3′ (encoding for WDTVTPEA and PEALGNL) for β-isoforms. All primers were designed from constant domains of the *Xenopus* isoforms. PCR products were separated on a 1.5% agarose gel and transferred to a Zetaprobe nylon membrane (Bio-Rad). After 4 h prehybridization at 60°C, the membrane was hybridized for 16 h with the labeled primer 5′GACACCATCTGTCTTCTTGTTC3′, encoding NKKTDGV for γ-isoforms, the labeled primer 5′TGGCTACCAGAAACTTCTCAGCGAAG3′, encoding ATRNFSA for δ-isoforms, and the labeled primer 5′GCCGGAGGAATGGGTCCCTTTGG3′, encoding PKGPIPPA for β-isoforms. Primers were labeled using T4-polynucleotide kinase (Fermentas) to a specific radioactivity of 3.10^6 cpm/ml. Membranes were washed four times for 10 min at 60°C with 6× SSC and 0.5% SDS, and the PCR-DNA fragments were visualized using a phosphoimager-intensifying screen. To reprobe the southern blot for γ-isoforms with the labeled primer (5′GCAAATCCTGTCTGTCCTGGGG3′) present in domain XII (see further), we dehybridized the blot first 3 times for 20 min in 0.1× SSC and 0.1% SDS at 100°C. The blot was further treated with the labeled primer as described above.

RESULTS

cDNA Cloning of CaMKII Isoforms from Xenopus laevis Prophase Oocytes—Cyk/CaMKII isolated from prophase *Xenopus* oocytes is a holoenzyme consisting of at least five different isoforms that migrate on SDS-PAGE as 52, 56, 62, 72, and 83 kDa proteins. At least three of them can be considered as new, since the highest molecular mass proteins (62, 72, and 83 kDa) contain a tryptic peptide (QTXAPVVAATSAANLV) showing no similarities to known CaMKII isoforms or any other protein (*10*).

To clone the cDNA of these new CaMKII isoforms from a *Xenopus* prophase oocyte λgt10 cDNA library, a two step approach was followed. In a first step a nested PCR strategy was followed to screen for the variable region of the different isoforms, essentially as described (*11*) (Fig. 1A). Species-specific primers were derived from the *Xenopus* β isoform (accession number U06636), in addition to the β′ isoform, the only *Xenopus* CaMKII isoforms known so far in the database. The nested PCR mixture obtained contained bands between 400–800 bp (Fig. 1B). The entire mixture was cloned directly into a pBluescript II SK⁻ vector without fractionation and positive clones were sequenced. This allowed us to align four different variable regions of, respectively, 753, 684, 513, and 447 bp. All clones contained sequences encoding for the so far unknown tryptic peptide QTXAPVVAATSAANLV present in the high molecular weight protein bands of *Xenopus* Cyk/CaMKII (62–83 kDa) (*10*). Thus, we were dealing with cDNA fragments encoding CaMKII isoforms present in our Cyk/CaMKII preparation (*10*). But, based on the molecular mass of the 83 kDa protein, we expected a maximal DNA fragment encoding for a variable region of about 1,100 bp. Therefore, we supposed that at least the 83 kDa protein would have a specific extension in its cDNA not common to other CaMKII isoforms.

In order to clone full-length cDNAs of the CaMKII isoforms, including the cDNA encoding the 83 kDa form, the *Xenopus* prophase oocyte λgt10 library was screened by the conventional filter screening method. To screen specifically for the higher molecular weight protein bands, a 200 bp

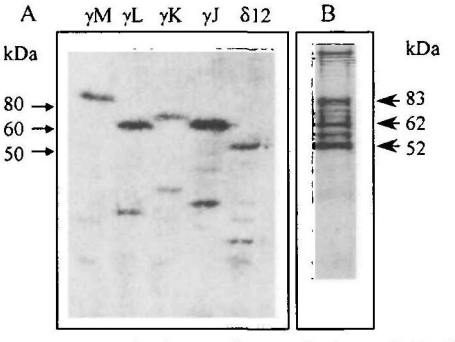

Fig. 2. ***In vitro* transcription and translation of cDNAs of different cloned isoforms.** Different cDNAs were translated in a rabbit reticulocyte lysate as described in "MATERIALS AND METHODS." A: Autoradiogram of [^{35}S]methionine-labeled translation products of the different *Xenopus* CaMKII isoforms after separation on SDS-PAGE. The position of some of the 10 kDa MW markers (Gibco, BRL) are indicated on the left. B: Coomassie staining of the SDS-PAGE of non-activated CaMKII, as isolated from prophase *Xenopus* oocytes. The molecular masses of some of the proteins were calculated in comparison with the 10 kDa marker ladder, and are indicated on the right.

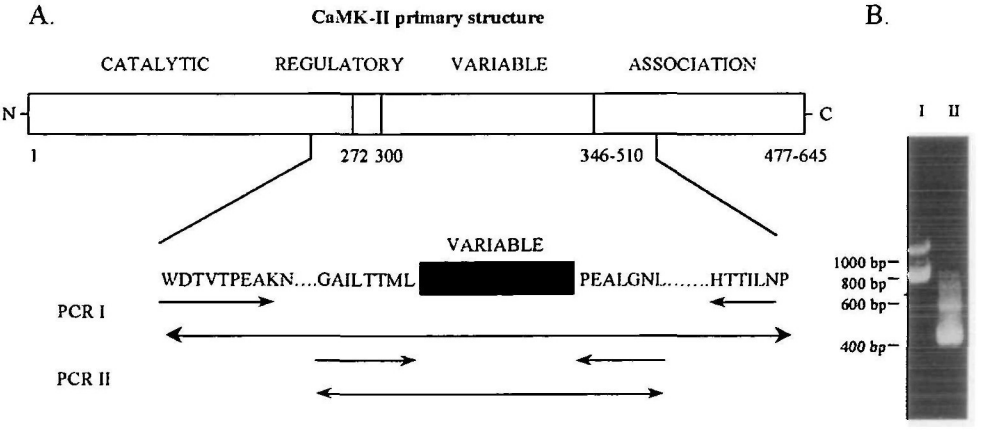

Fig. 1. **The nested PCR strategy to amplify variable domains of CaMKII isoforms.** A: Schematic representation of the primary structure of CaMKII isoforms. Each contains a catalytic, regulatory, variable, and association region. The sequences of the domains flanking the variable region are shown, and the primers used in the two consecutive PCR reactions (PCR I, PCR II) are indicated by arrows. B: PCR reactions were performed using a prophase *Xenopus* oocyte λgt10 cDNA library as template, and the resulting PCR products of PCR I and PCR II were visualized on an agarose gel.

probe of the variable region was used comprising sequences encoding for the unknown peptide mentioned above. In an attempt to cover the whole range of CaMKII isoforms, the same library was rescreened with a probe containing part of the constant domains of the CaMKII isoforms (autophosphorylation and calmodulin binding region). This probe was made by PCR using the cDNA of the *Xenopus* β isoform as a template. Using this screening strategy, 10 positive clones were isolated, representing five different full-length cDNAs [accession numbers: AF233629 (δ12) (3 clones), AF233630 (γJ) (2 clones), AF233631 (γK) (1 clone), AF233632 (γL) (1 clone), AF233633 (γM) (3 clones)]. These cDNAs were cloned into pBluescript II SK⁻ vector and sequenced (for the nomenclature used, see further). Four of the five isolated clones had similar 5′ and 3′ untranslated regions. Three had an incomplete 3′ non-coding sequence that stopped at an internal *Eco*RI site (AF233630, AF233631, AF233633), whereas the other one (AF233632) had a full-length 3′ non-coding sequence with several common (AATAAA) and uncommon (ATTAA) polyadenylation signals (*15*). One clone (AF233629) had totally different endings, suggesting that it is derived from a different gene. No DNA extensions outside the variable region were found. The cDNAs encode proteins with calculated molecular masses of 71.5 kDa (γM), 68.9 kDa (γL), 63.0 kDa (γK), 60.6 kDa (γJ), and 53.9 kDa (δ12). However, *in vitro* transcription and translation reactions of these clones with the appropriate RNA polymerases, and visualization of the [^{35}S]methionine labeled proteins by autoradiography revealed proteins migrating as 83 kDa (γM), 62 kDa (γL), 68 kDa (γK), 60 kDa (γJ), and 52

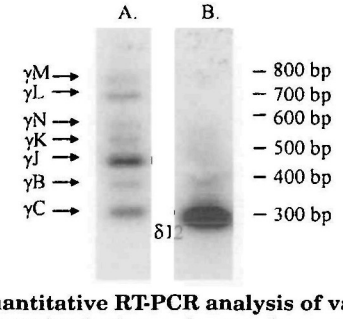

Fig. 3. **Semi-quantitative RT-PCR analysis of variable regions of different CaMKII isoforms in prophase oocytes.** RT-PCR products encompassing the variable regions were separated on 1.5% agarose, blotted and hybridized with specific oligonucleotides for γ-isoforms (A) and δ-isoforms (B) as described in "MATERIALS AND METHODS." Arrows on the left indicate the corresponding CaMKII isoforms. Migration of the 100 bp DNA marker (GIBCO, BRL) is indicated on the right.

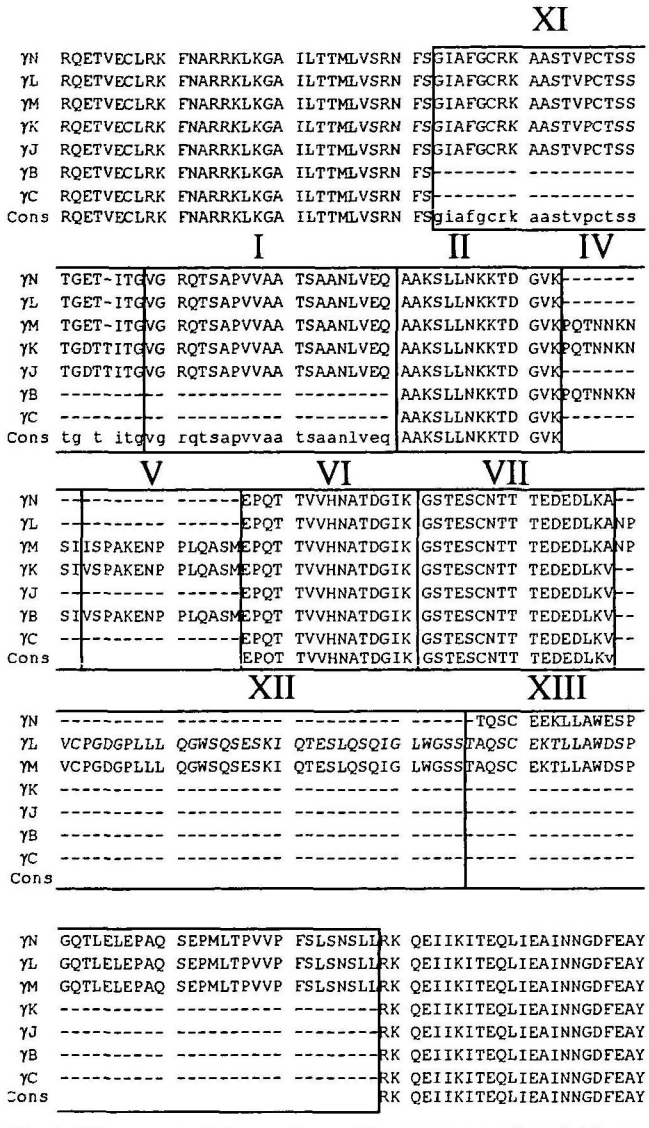

Fig. 4. **Alignment of the amino acid sequences of variable regions of different CaMKII-γ-isoforms.** The variable regions of the different CaMKII isoforms (γJ, γK, γL, γM, γB, γC, γN) are aligned. The different domains delineated by Tombes and Krystal (*11*) are boxed and shown by Roman numerals above the amino acid sequences. The new domains (domain XI, XII, XIII) are named to fit into this nomenclature. Absolute conserved residues are listed below the sequences.

TABLE I. **Different features of isolated CaMKII isoforms from prophase *Xenopus* oocytes.**

Name	Accession number	Domains in variable region	cDNA[a]	Molecular mass (kDa)[b]
γJ	AF233630	IX, I, II, VI, VII	FL	60.6 (P)–62 (E)
γK	AF233631	IX, I, II, IV, V, VI, VII	FL	63.0 (P)–68 (E)
γL	AF233632	IX, I, II, VI, VII, XII, XIII	FL	68.9 (P)–60 (E)
γM	AF233633	IX, I, II, IV, V, VI, VII, XII, XIII	FL	71.5 (P)–83 (E)
γN	AF233634	IX, I, II, VI, VII, XIII	VR	65.1(P)
γB	AF233635	II, IV, V, VI, VII	VR	58.5 (P)
γC	AF233636	II, VI, VII	VR	56.1 (P)
δ12	AF233629	II, VII	FL	53.9 (P)–52 (E)

[a]FL: full length cDNA, VR: only variable region. [b](P) predicted from their amino acid composition (Swiss Prot, MW calculation) or (E) experimentally found by migration of the *in vitro* translated products on SDS-PAGE.

kDa (δ12) proteins in SDS-PAGE (Fig. 2). The proteins with calculated molecular masses of 71.5 and 63.0 kDa migrated, respectively, as 83 and 68 kDa proteins in SDS-PAGE, whereas the protein γL, with a calculated molecular mass of 68.9 kDa, migrated as a 62 kDa protein. This demonstrates that the molecular mass of the primary sequence is not the only determinant for migration in SDS-PAGE, and that other features, such as hydrophylicity, resulting in lower SDS binding, exposure of other domains to the molecular surface, or posttranslational modification might result in "abnormal" migration in SDS-PAGE.

After comparing the five *in vitro* translated protein bands with the proteins present in purified *Xenopus* prophase oocyte CaMKII, we suspected that not all cDNAs were cloned. The cDNAs encoding the 56 kDa and 72 kDa proteins, both present in lower quantities in purified CaMKII, could perhaps not be identified, although phosphorylation could cause an extra upshift of the isomers in the CaMKII preparation as isolated [especially the higher molecular weight forms (*10*)] that might be slightly different from isomers synthesized in reticulocyte lysates. To identify the remaining isoforms in the CaMKII preparation, a RT-PCR strategy was followed. A reverse transcriptase reaction was performed using a primer containing the translation stop of the δ- and γ-isoforms to obtain some specific cDNA. After purification of this cDNA, semi-quantitative PCR was performed with primers present in the constant domains flanking the variable region of the δ- or γ-isozymes. PCR-products were visualized on Southern blots, which were hybridized with internal primers derived from the δ- and γ-isozymes (Fig. 3). Using this strategy three other isoforms could be identified (γB, γC, γN).

Comparison of the amino acid sequences predicted from the different isolated cDNAs showed that all isoforms differ almost exclusively in their variable regions. An alignment of the variable regions of the different isolated clones is shown in Fig. 4. The variable regions can be subdivided into different domains (exons) similar in all known CaMKII isoforms (*11, 16*). These domains are named by roman numbers according to the nomenclature introduced by Tombes and Krystal (*11*). Seven of the isolated clones of *X. laevis* oocytes have a variable region containing at least domains II, VI, and VII, almost identical to the corresponding domains of γ-isoforms in other species, and are therefore denoted as γ-isoforms. Still, some minor differences, most probably due to species differences, were also found in these domains. Two isoforms could be identified as similar to γB and γC, previously described in other species (*17*). The other isoforms contain a novel domain in front of domain I that we denoted as domain XI. Finally some isoforms contain one or two new domains after domain VII that we denoted as domains XII and XIII. Overall, it appears that the five new isoforms are splice variants of the γ-gene containing one, two or three novel domains. We designated these isoforms as γJ, γK, γL, γM, and γN in line with the nomenclature used so far for γ-variants (*11*). The variable region of one isoform showed the highest similarity to the known CaMKII δ-isozyme family. The variable region contained only domains II and VII with sequences identical to those of CaMKII δ-isozymes, and no C-terminal extension was found as described for some other δ-isoforms (*18*). This isoform showed the closest similarity to δ6. Only one alanine was missing at the N-terminus of domain II. Such an isoform was also found in rabbit liver by Takeuchi and Fujisawa (*16*) and was denoted as the δ12 variant. A summary of the different features of the isoforms is provided in Table I.

Expression of the Different Isoforms in Xenopus Adult Tissues—Because of the presence of a whole range of different new isoforms in prophase *Xenopus* oocytes, we wondered whether these isoforms are only present during the

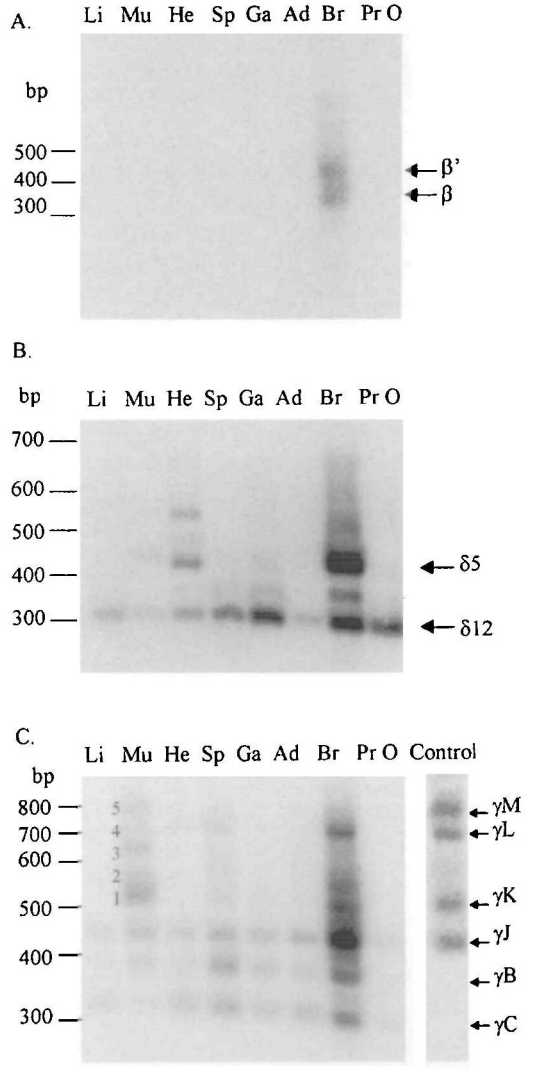

Fig. 5. **Expression of the different CaMKII isoforms in different adult tissues.** Variable regions of β-isoforms (A), δ-isoforms (B), and γ-isoforms (C) were amplified using semi-quantitative RT-PCR. cDNA of different isoforms was prepared from 2 μg total RNA using reverse transcriptase and the corresponding "stop" primer. A PCR reaction was performed with specific primers for the different isoforms that flanked the variable region. To be in the linear amplification range, only 20 amplification cycles were performed. RT-PCR products encompassing the variable regions were separated on 1.5% agarose, blotted and hybridized with the specific primers for each isoform. The expression of the different isoforms was measured in liver (Li), muscle (Mu), heart (He), spleen (Sp), gall bladder (Ga), adipocytes (Ad), brain (Br), and prophase oocytes (Pr. O.). The control lane represents the PCR amplification of the variable region of the different cDNAs of isolated γM, γL, γK, and γJ from the λgt10 oocyte library. Bands indicated by numbers in C represent γK (1), γO (2), γP (3), γL (4), γM (5), as identified by a domain XII–specific probe and sequencing (see text).

early development of *X. laevis* and not in adult tissues after differentiation. Therefore, we compared the expression patterns of the different isoforms in several adult tissues and in prophase *Xenopus* oocytes. Because of the low abundance of CaMKII mRNAs in oocytes and some other adult tissues, a northern blot strategy was impossible. We chose the semi-quantitative RT-PCR strategy already described for the identification of all isoforms in prophase *Xenopus* oocytes. Total RNA from several adult tissues (liver, muscle, heart, spleen, gall bladder, adipocytes, and brain) was used as a template.

Southern blots demonstrating the expression pattern of different isoforms (β, δ, γ) in these adult tissues are shown in Fig. 5, A, B, and C.

The β-isoform and the β′-isoform are so far the only *Xenopus* isoforms described in the genbank database. The origin

TABLE II. **Sequences of domains XII and XIII in the variable region represented in Fig. 6.**

Domain number	Sequence
XII	NPVCPGDGPLLLQGWSQSESKIQTESLQSQIGLWGSSM
XIII	TAQSCE-KTLLAWDSPGQTLELEPAQSEPMLTPVVPFSLSNSLL
XIII′	T-QSCEEK-LLAWESPGQTLELEPAQSEPMLTPVVPFSLSNSLL

Note: Domain XIII is found only in those isoforms that also have domain XII in the variable region. Domain XIII′ always links immediately to domain VII.

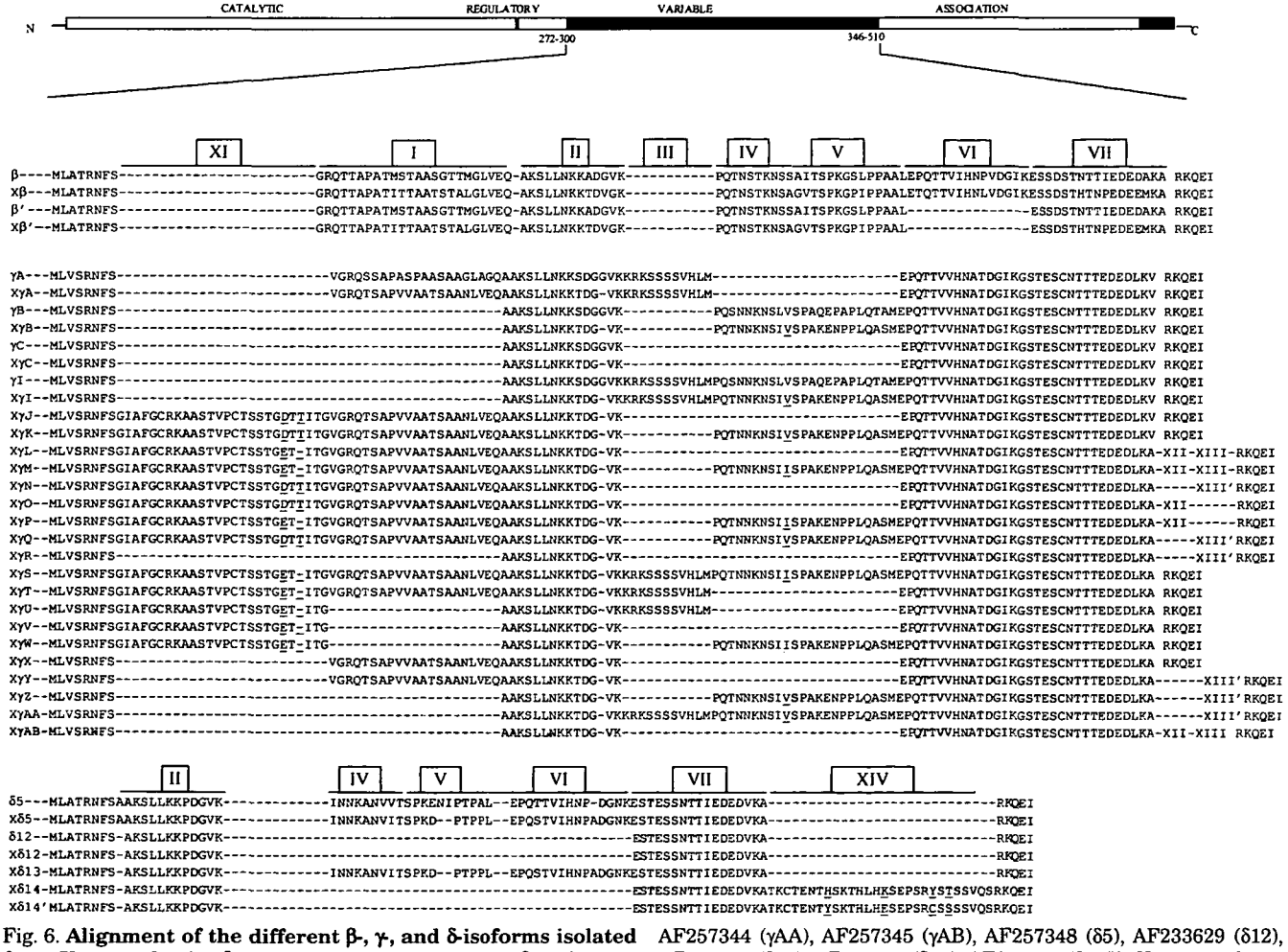

Fig. 6. **Alignment of the different β-, γ-, and δ-isoforms isolated from *Xenopus* brain (β- and γ-isoforms) and heart (δ-isoforms).** A schematic representation of the CaMKII structure of the different isoforms is presented at the top of the figure. An alignment of the variable region of the different *Xenopus* (prefix "X") isoforms is shown below (accession numbers: U06636 (β), U18196 (β′), AF257346 (γA), AF233635 (γB), AF233636 (γC), AF257347 (γI), AF233630 (γJ), AF233631 (γK), AF233632 (γL), AF233633 (γM), AF233634 (γN), AF257332 (γO), AF257333 (γP), AF257334 (γQ), AF257335 (γR), AF257336 (γS), AF257337 (γT), AF257338 (γU), AF257339 (γV), AF257340 (γW), AF257341 (γX), AF257342 (γY), AF257343 (γZ), AF257344 (γAA), AF257345 (γAB), AF257348 (δ5), AF233629 (δ12), AF257349 (δ13), AF257350 (δ14), AF257351 (δ14′)). Known isoforms (β, β′, γA (*4*), γB, γC (*17*), γI (*16*), δ5 (*18*), δ12 (*16*)) are compared with their mammalian counterparts. Different domains are indicated by Roman numerals according to the nomenclature introduced by Tombes and Krystal (*11*), and are indicated in boxes above the amino acid sequences. The new domains in *Xenopus* isoforms are named similarly. Sequences of the domains, which are not represented, are summarized in Table II. Small sequence differences within the different domains of *Xenopus* isoforms that arise due to the tetraploid characteristic of the *Xenopus* species are underlined.

of the isoform U06636 is listed as ovary, but it is doubtfull that this β-isoform originated from oocytes rather than from other ovarian cells, such as follicular cells. During several rounds of screening of the *Xenopus* oocyte λgt10 library with a β-specific probe, we never isolated this isoform. As shown in Fig. 5A, also by using the RT-PCR strategy, we found the expression of the β- and β′-isoforms only in brain and not in other tissues. These data confirm the observation of Tobimatsu and Fujisawa (*4*) who also could demonstrate only α- and β-isoforms in rat neuronal tissues.

The expression pattern of the δ-isoforms is shown in Fig. 5B. The δ12 isoform, the only δ-isoform expressed in oocytes, is expressed in all tissues, with the highest expression in brain and gall bladder. Muscle, heart and brain express also another δ-isoform in addition to the δ12 isoform. The DNA fragment of this isoform was cloned in a pBluescript II SK⁻ vector and sequenced. It was identified as the δ5 isoform described earlier (*18*). The δ5 isoform is expressed in muscle, heart and brain. We also isolated a variant of δ5 in brain that has only one alanine at the beginning of domain II, as in the δ12 isoform described for the first time by Takeuchi and Fujisawa (*16*). This isoform has never been described before, and we call it the δ13 isoform. In heart, we isolated another novel δ-isoform that possesses a novel domain never described before in δ-isoforms, in addition to the known domains II and VII. This novel domain is called domain XIV and the isoform δ14.

The distribution of the different γ-isoforms is shown in Fig. 5C. Different isoforms are expressed most abundantly in brain and the amount of γ-isoforms in prophase oocytes is lower than in adult tissues. The γ-isoforms expressed in prophase oocytes are also expressed in adult tissues. γB, γC, and γJ are ubiquitous and γJ shows the highest expression in brain. γL and γM are also expressed in all tissues, but the expression is remarkably higher in muscle and brain. Other tissues show lower expression of these isoforms. γK displays the highest expression in muscle (represented as band number 1). To identify the remaining isoforms in muscle we reprobed the southern blot with a labeled primer specific for domain XII. The four upper bands interacted with this probe (represented in Fig. 5C as 2-5). After sequencing these bands we identified them as γL and γM (4 and 5), also described in prophase oocytes, and two other novel isoforms that we called γO and γP (2 and 3). The latter two isoforms contain either domains XI, I, II, VI, VII, XII (γO) or domains XI, I, II, IV, V, VI, VII, XII (γP) in their variable regions. On the other hand, brain tissue not only shows the highest expression of the different isoforms described earlier in prophase oocytes, but also expresses a range of other isoforms. To identify all the isoforms expressed in brain, all of the DNA fragments generated by PCR were cloned in pBluescript II SK⁻ vector and sequenced. A summary of all isoforms isolated from brain is shown in Fig. 6. Besides some known isoforms such as γA, γB, γC, γI, many new isoforms were isolated due to the presence of one, two or three new domains (domain XI, XII, XIII) in their variable regions, domains that were also found in the new isoforms characterized in prophase oocytes. A total of 14 new isoforms were isolated that were not found in prophase oocytes and named according to the nomenclature used so far (γO-γZ, γAA, γAB). The new isoforms are represented in Fig. 6 with their specific domains in the variable region.

DISCUSSION

There is growing evidence for a function of CaMKII in the activation process of *Xenopus* oocyte maturation. A constitutive active mutant of CaMKII-β can initiate maturation (*8*), and inhibitors BAPTA (a specific calcium chelator) and AIP (a specific CaMKII inhibitor) block or retard maturation (*10*). Moreover, the activation of CaMKII during oocyte maturation has been demonstrated by *in vitro* (*9*) as well as *in vivo* (*10*) assays. At fertilization, the CDK1 regulatory cyclin subunit is degraded by an elevation of cytosolic calcium (reviewed in Ref. *19*), and a specific Ca^{2+}, calmodulin mediated event is responsible for cyclin degradation (*20*). Later it was shown that the target for Ca^{2+}, calmodulin is CaMKII (*6, 21*). Both observations indicate that CaMKII plays a role in the activation (during oocyte maturation) as well as in the inactivation (during fertilization) of CDK1. Furthermore, Lindsay and coworkers (*22*) showed, by the addition of dibromo-BAPTA to mitotic extracts at different time points, that local transient elevation in free Ca^{2+} is necessary for both the activation and inactivation of CDK1. This phenomenon can be explained by the ability of CaMKII to decode the frequency of calcium spikes (*23, 24*). At a low frequency of calcium spikes, CaMKII reaches only submaximal activity, which might result in a different *in vivo* function.

Recently, CaMKII was isolated from *Xenopus* prophase oocytes (*10*), and identified as a holoenzyme comprising at least five proteins with molecular masses ranging from 52–83 kDa. All could be identified as isoforms of CaMKII, and, after peptide sequencing, some were found to be novel. Because of the important role of CaMKII during *Xenopus* oocyte maturation, it became necessary to characterize *Xenopus* CaMKII at the molecular level. Since only cDNA of *Xenopus* β and β′-isoforms was known, we started with the molecular cloning of these isoforms from a *Xenopus* oocyte cDNA library. The cDNAs of five different full length CaMKII isoforms were isolated: four could be identified as novel splice variants of the γ-gene that we named γJ-M and one as the δ12 isoform, previously shown to be present in rabbit liver (*16*). *In vitro* transcription/translation of these five full length isoforms and SDS-PAGE of the resulting proteins allowed us to provisionally identify the 83 kDa, 68 kDa, and 52 kDa CaMKII isoforms in the oligomeric holoenzyme as the gene products of, respectively, γM, γK, and δ12. The 62 kDa band most probably represents a mixture of the proteins encoded by γL and γJ since the *in vitro* transcription/translation products of both clones migrate at about the same molecular mass in SDS-PAGE. The 72 kDa protein is possibly also encoded by γK. After autophosphorylation of the isolated Cyk/CaMKII preparation, the 72 kDa band of the 68/72 kDa protein doublet was more pronounced, and in the ATP-γ-S activated preparation, only the 72 kDa protein could be observed (see Fig. 3 in Ref. *10*), indicating that the 72 kDa protein is most probably the result of post-translational modification.

Since we had no ready explanation for the 56 kDa protein band in the holoenzyme, we suspected that not all CaMKII isoforms present in *Xenopus* prophase oocytes were cloned. Therefore we used a second screening strategy to identify the remaining CaMKII isoforms. RT-PCR starting from total RNA of prophase oocytes demonstrated the

presence of one additional novel γ–isoform (γN), along with two known γ–isoforms (γB and γC). The latter three isoforms exist only as clones of the variable region. Assuming no variation in their constant domains, the full length clones are predicted to encode proteins of 65.1 (γN), 58.5 (γB), and 56.1 (γC) kDa. However, since it is clear from the *in vitro* transcription/translation experiment of full length clones that the presence of a different set of exons in the variable region can cause up shifts as well as down shifts in the expected migration in SDS-PAGE, it is difficult to predict the migration of the gene products of γN, γB, and γC in SDS-PAGE. One probably codes for the protein migrating at the 56 kDa position, and the others probably contribute to the other protein bands present in the holoenzyme (possibly also the 72 kDa protein). Therefore, although it is not possible to determine with certainty which isolated clone encodes for which protein in the holoenzyme, the eight different cDNA clones most probably cover all isoforms present in the CaMKII holoenzyme of *Xenopus* prophase oocytes.

A semi-quantitative RT-PCR of the variable regions starting from total RNA of prophase and metaphase II oocytes, and Southern analysis with γ and δ isoform-specific probes (as described for prophase in Fig. 3) revealed no difference in the expression of one of the eight mRNAs (results not shown). This result underscores the hypothesis that it is rather a rise in local Ca^{2+} than the induction of a specific isoform that forms the basis of CaMKII activation during maturation.

Injection of the messenger for a constituve active mutant of the γM isoform (encoding the 83 kDa isoform with a Thr287 mutation to an aspartic acid) induced maturation in the absence of progesterone (Rondelez, E., Stevens, I., and Goris, J., unpublished results). A similar observation was already made by injecting the messenger for a constitutive active mutant of the β-isoform (*8*). Since no such isoform is expressed in oocytes, it can be expected that the other isoforms will do the same. However, the mutation of Thr287 to aspartic acid turning CaMKII into a Ca^{2+}-independent form was essential for maturation. This probably reflects the fact that activation through an endogenous mechanism (rise in Ca^{2+}) resulting in autoactivation is still essential for the activation of the wild type protein.

The presence of only δ- and γ-isoforms in *Xenopus* prophase oocytes could be expected from their non-neuronal origin, but a totally novel spectrum of CaMKII isoforms was observed in comparison with mammalian tissues. Originally, it was tacitly accepted that the molecular mass of the different isoforms was between 50–65 kDa. Higher molecular mass isoforms had never been observed. Moreover, higher molecular mass bands observed after nested PCR (*16*), or after purification of CaMKII from rat heart (*25*) were previously considered to be "too high" and therefore described as "aspecific bands not analyzed." The high molecular weight of the newly identified isoforms sheds new light on the molecular weight spectrum of the CaMKII isoforms and gives definitive credibility to CaMKII isoforms with molecular mass higher than 65 kDa.

Unlike in other species, the isolated γ-isoforms are probably splice variants of two genes. The clones differ not only in their variable regions, but also show minor differences scattered over the catalytic, regulatory, and association regions. This peculiar genetic feature can be explained by the tetraploid characteristic of *X. laevis*. During the development of the *X. laevis* genus 30 million years ago, a duplication of the genome occurred. Since normal evolutionary mechanisms might have operated differently on the two copies, this may have resulted in some minor structural variations in the protein in an individual animal (*26*).

To determine the potential role of the new isoforms in the early development of *X. laevis*, we also examined the expression pattern of the new isoforms in adult tissues. A semi-quantitative RT-PCR strategy was used because of the low abundance of these isoforms. All isoforms isolated from prophase oocytes were also found to be expressed in adult tissues. Moreover, other tissues, such as brain and muscle, contain a whole range of new γ-isoforms, as well as some new δ-isoforms in addition to the isoforms isolated from oocytes.

Besides the known domains, other domains not previously identified in other CaMKII isoforms are also present. In γ-isoforms, one domain is present in front of domain I, which we named domain XI. Two domains are present after domain VII in front of the association region–like domain X in the β3-isoform (*27*), and these were named domains XII and XIII. We also isolated two novel isoforms from heart, muscle and brain, δ13 and δ14. Delta 13 is an isoform identical to δ5 but missing one alanine (as in the δ12 isoform) at the beginning of domain II. One δ-isoform isolated from heart contains one novel domain in addition to the known domains II and VII, which we called domain XIV.

The function of these new domains is not yet known; no sequence similarities were found with other proteins and analysis of the new domains for consensus sequences (swiss-prot, prosite) was negative. These novel domains (XI, XII, XIII) were found in oocytes as well as in adult tissues. Therefore, the splice mechanisms that govern the appearance of these isoforms are not specific to early embryonic development. Since the novel domain XIV in the δ-isoforms is found only in heart and skeletal muscle, it might have a specific function in these organs. In fact, little is known about the specific function of the different domains (exons) in the variable region. So far, domain I had been found only in CaMKII isoforms of neuronal origin (*11*). Since our results clearly demonstrate the presence of domain I in all novel γ-isoforms from oocytes, the function of this domain is certainly not limited to neuronal signal transduction. Domains II and VII are present in all CaMKII isoforms identified so far, and, therefore, are considered to form a conserved tether between the regulatory and association regions. Domain III is known to contain the functional nuclear localization signal KKRK, homologous to the nuclear localization signal of the SV40 large T antigen. CaMKII holoenzymes with the right proportion of isoforms containing domain III in their variable region are targeted to the nucleus (*28*), and phosphorylation of the serine next to the nuclear localization signal blocks nuclear targeting (*29*). Since these isoforms are able to phosphorylate transcription factors CREB and ATF-1, they have been thought to have a function in gene expression (*29*). Also in *Xenopus* brain, some isoforms were isolated containing domain III in their variable regions. Electron microscopic studies of the CaMKII holoenzymes revealed that the holoenzyme is arranged in a hub- and spoke-pattern. The N-terminal catalytic/regulatory domains of each subunit extend out as

spokes that are tethered by the variable domain to a large central hub region composed of the C-terminal-association domains (*1, 30*). Therefore, it is suggested that inserts in the N-terminal half of the variable domain, which is closest to the catalytic/regulatory domain, will have an effect on the calcium, calmodulin binding, calmodulin trapping, or substrate specificity of the enzyme (*25*). Since domain XI is closest to the calmodulin-binding site, it is possible that it affects the calmodulin binding of the isoforms. Insertions in the C-terminal half would be part of the central hub region and could serve to regulate the multimer size of the kinase and its targeting to intracellular sites. For example, in the sarcoplasmic reticulum, an interaction was demonstrated between αKAP and catalytically competent CaMKII isoforms. αKAP is an anchoring protein for CaMKII encoded by a gene within the gene of αCaMKII (*31*). Anchoring to the sarcoplasmic reticulum brings CaMKII near the entry site of calcium, and increases its concentration near physiological substrates, such as the ryanodine receptor, phospholamban and the calcium pump protein. Interaction is shown through the association domain of CaMKII (*31*). Domains in the C-terminal half of the variable region, close to the association domain should influence such interactions. A nice example of a possible function in the interaction with other proteins is given for the β3-isoform isolated from a pancreatic β-cell (*27*). This isoform contains two domains in tandem near the association domain, each of which contains a proline-rich region and a putative SH3 binding sequence. Such proline-rich regions are found in many proteins and have been implicated in the reversible association of proteins into functional complexes regulated by phosphorylation within the proline-rich region (*32*), whereas SH3 binding sequences have been implicated in many signal transduction pathways (recently reviewed in Ref. *33*). It has been found (*11*) that tumor cells, including those of neuronal origin, express an entirely different spectrum of CaMKII isoforms than adult neuronal tissues. This finding has been taken as support for a role of CaMKII in growth control (*11*).

The expression of the different *Xenopus* isoforms in prophase oocytes and in adult tissues provides insight into the diversity of the different CaMKII isoforms both within and between different species. The primary goal of our research is to understand the regulation of the cell cycle, and further research is necessary to dissect the specific functions of the different exons in the plethora of new CaMKII isoforms in recognizing specific substrates during the G2/M transition. Meanwhile the structural basis is laid with which to start this complex task.

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