Developmental expression of the creatine kinase isozyme system of *Xenopus*: maternally derived CK-IV isoform persists far beyond the degradation of its maternal mRNA and into the zygotic expression period

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Summary

The differential expression of the multilocus CK isozyme system throughout development of the two *Xenopus* species *X. laevis* and *X. borealis* was investigated. A cDNA containing the nearly complete coding sequence of the CK-IV subunit of *X. laevis* was isolated and sequenced. Early development of *X. laevis* proceeds with a stock of maternally derived CK-IV/IV isozyme. While the mRNA declines rapidly after fertilization and disappears before neurulation, maternal CK-IV/IV isozyme is active far beyond the onset of zygotic expression and is still detectable when tadpoles start feeding. Zygotic expression of CK-IV begins after neurulation, at stage 22/24, and seems to start simultaneously with that of another gene, CK-III. Modulation in the expression of these two genes and the appearance of two other isoforms, the CK-I and CK-II/III isozymes, take place during development in a tissue-specific manner. During metamorphosis, the CK phenotypes of eyes and skeletal musculature undergo additional changes. The final adult pattern only appears several weeks after metamorphosis. The presumed orthologous CK isozymes of *X. borealis* show a developmental profile similar to that of *X. laevis*, except that CK-II/II is equally present in oocytes and during early development, in addition to CK-IV/IV isozyme. These results show that the expression of each of the four CK genes of *Xenopus* is under differential developmental control.

Key words: *Xenopus*, creatine kinase isozymes, zygotic expression, maternally derived.

Introduction

Creatine kinase (CK) enzymes catalyse the reversible phosphorylation of creatine from ATP and are thought to play a central role in energy metabolism. In higher vertebrates, two genes code for two different subunits, B (brain) and M (muscle), that combine into three dimeric cytoplasmic isozymes (MM, MB, BB). A mitochondria-associated isozyme, the Mi-CK (Jacobs et al. 1964) is encoded by a different gene (Hossle et al. 1988; Hass et al. 1989) and has an octameric structure (Schlegel et al. 1988). The tissue specificity (Eppenberger, 1983), developmental profile (Eppenberger, 1964) and genetic organization (Rosenberg et al. 1981) of CK isozymes have been extensively investigated in mammals, chicken and fish. Several amphibian species have been shown to possess a comparable CK isozyme system (Eppenberger et al. 1967; Fisher et al. 1980; Klemann and Pfohl, 1982), including a mitochondrial CK form (Legssyer and Arrio-Dupont, 1988).

In *Xenopus*, however, the CK zymograms reveal a more complex system that resembles the pattern found in teleost fish (Fisher and Whitt, 1978; Whitt, 1981) rather than that of tetrapods. By means of allelic polymorphism, at least four different CK genes have been identified for *X. laevis*, all of which show tissue-specific expression (Wolff and Kobel, 1985). The CK-I isozyme is found essentially in the eye and stomach and possibly represents a Mi-CK isozyme. CK-II/III and CK-IV/IV follow tissue-specific distributions comparable to that of the M-CK and B-CK of mammals and chicken or to the CK-A and CK-C of fishes, respectively. Adult skeletal muscles where CK-II/II is the main isozyme, as well as some other tissues of *Xenopus*, contain an additional isozyme, CK-III/III, whose orthologous relationship remains undetermined. CK-III and CK-IV subunits form homo- and heterodimers with each other *in vivo*, and also form heterodimers with rabbit M-CK subunits *in vitro* (Robert and Kobel, 1988), whereas CK-II/III isozyme seems to represent a
heterodimer between a CK-III subunit and a CK-II specific subunit (Bürki, 1985; Robert and Kobel, 1988). CK-II subunits do not form either homodimers or heterodimers with CK-IV subunits. Since most Xenopus species are of allopolyploid origin (reviewed by Kobel and Du Pasquier, 1986), the CK system of the tetraploid-derived X. laevis could reflect the conserved activity of one or other duplicated CK gene; yet, the diploid X. tropicalis displays an equally complex CK zymogram and other ancient tetraploid species, e.g. X. borealis and X. gilli, have additional CK isozymes compared to X. laevis that may represent such duplication.

In ontogenesis of higher vertebrates, CK isozymes show striking differential regulation. For example, the differentiation of myoblasts to skeletal and heart muscles is accompanied by a switch from BB-CK to MM-CK (Lough and Bischoff, 1977; Eppenberger et al., 1983), the latter becoming partly associated with the M line of myofibrils (Turner et al. 1973). The specific expression of M-CK isozymes has recently been shown to depend on an enhancer element (Jaynes et al. 1988). B-CK regulatory elements that respond to differentiation signals in cell types like monocytes or may be under the control of steroid hormones have also been described (Reiss and Kaye, 1981; Loike et al. 1984).

The multilocus system for CK isozymes of polyploid Xenopus is of particular interest: redundant genes may provide another means for biochemical refinements that, in higher vertebrates, seem to be achieved through complex splicing mechanisms from single CK genes (Bentfield et al. 1988; Wirz et al. 1989). The present study reveals that development of X. laevis zygotes proceeds with a large stock of maternally derived CK isozymes and it is not until embryonic stage 24 (tailbud, 15 somites, motor reaction to external stimulation) that a novel CK isozyme, i.e. CK-III/III, becomes discernible. In growing tadpoles, individual organs display different patterns of CK isozymes that have built up during organogenesis. Zymograms of larval organs can be performed on cellulose acetate membranes (Titan III, Helena) at 200 V for 30 min, in a 75 mM Tris (pH 7.5), 3 mM EDTA, 14 mM citric acid buffer. Membranes were stained for CK activity according to Harris and Hopkinson (1976) with the addition of P$_2$P$_5$-di(adenosine 5')-pentaphosphate (0.1 mM) in order to inhibit adenylate kinase (Lienhard and Secemski, 1973).

Isolation and characterization of cDNA clones

We have screened at low stringency a Charon 4A X. laevis genomic library with chicken probes derived from B-CK and M-CK cDNA clones. Several clones were isolated, one of which was used to screen a X. laevis cDNA library from stage 40 larvae. Various cDNA and genomic DNA fragments were subcloned in pEMBL 8 and 9 vectors and grown as single-strand template (Dente et al. 1983) with helper phage M13K07. Sequence analysis was performed by the standard dideoxy chain termination method (Sanger et al. 1977) using Sequenase enzyme (USB).

RNA isolation and analysis

Total cellular RNA was isolated from pools of 200 embryos at different stages, and from different organs of adult X. laevis by the Li/urea procedure (Aufray and Rougeon, 1980). Samples of 10 µg RNA were denatured with glyoxal (Thomas, 1983), resolved on 1% agarose gel and transferred to nylon membranes. Hybridization was performed for 48 h at 42°C with 32P-labeled pX21 fragment, a PsI 500 bp digest from pBR2 cDNA containing the sequence of CK-IV/IV subunit. The sizes of mRNA were determined using RNA marker (Boehringer). We verified that each lane of the blot contains equal amounts of RNA by reprobing the membrane with a X. laevis cDNA probe containing the entire coding region (G. Spohr, personal gift).

Results

Isolation and characterization of a CK-IV cDNA clone

Using two cDNA probes from chicken B-CK (Hossle et al. 1986) and M-CK (Ordhal et al. 1984) at low stringency, several clones were isolated from a X. laevis genomic library (Stutz and Spohr, 1986; see Materials and methods). One of them, pGX42, was further characterized. Partial DNA sequencing revealed homology to chicken B-CK and this genomic fragment was used to screen a cDNA library constructed from stage 49 larvae by G. Spohr (personal gift). Fig. 1 shows the DNA sequence of the cDNA clone carrying the largest insert, pXCK1, and its deduced amino-acid sequence.
The position of an intron found by sequencing a fragment of the genomic DNA corresponds to position 61 in the complete primary structure of chicken B-CK. The active site (residues 268-292) is marked with an arrow.

**Fig. 1.** Nucleotide and deduced amino acid sequence of the pXCKl cDNA clone of X. laevis. The first nucleotide identity with B-CK and 84% with M-CK of chicken, whereas comparison with the CK sequences of two species shows 82% amino acid sequence identity. The C-terminal sequence Pro-Ala-Gln-Lys, common to all known CK sequences (Babbit *et al*. 1986), as well as the Cys residues of the active site are equally conserved in this CK gene of *Xenopus*. Preliminary sequencing of genomic fragments (data not shown) suggests evolutionary conservation among CK genes of different species since an intron at position 910 in this CK coding sequence is found at exactly the same location in the rat B-CK and M-CK genes (Bentfield *et al*. 1988). Moreover, the coding sequence of the genomic fragment pGX42 is strictly identical with that of pXCK1 cDNA.

Northern blot analysis (Fig. 2) shows that pXCK1 recognizes a 1.6 kb RNA present in embryos, ovaries and some other adult tissues, but absent in adult skeletal muscle. This distribution agrees with the tissue specificity of the CK-IV/IV isozyme (Wolff and Kobel, 1985). For example, adult skeletal muscles express CK-IV.

**Fig. 2.** Northern blot analysis of total cellular RNA isolated from pools of 200 embryos at different development stages and from different organs of adult *X. laevis*. Hybridization was performed for 48 h at 42°C with 32P-labeled pX21 fragment, a 500 bp digest from pXCK1 cDNA, containing the nearly complete sequence of a CK-IV subunit. The size of mRNA was determined using RNA markers (Boehringer). We verified that each lane of the membrane with a *X. laevis* rDNA probe containing the entire coding region.
II/III and CK-III/III but no CK-IV/IV, whereas ovary contains only the CK-IV/IV isozyme.

In addition, a restriction fragment length polymorphism (RFLP) was detected, using the pGX42 genomic probe, in gynogenetic offspring from a female heterozygous for the fast and slow allozymes of both CK-IV/IV and CK-II/III (unpublished data). The two RFLP alleles showed complete cosegregation with either the fast or the slow CK-IV allozyme. In contrast, they showed independent segregation with respect to the polymorphic CK-II subunit. From these data, it follows that the pGX42 sequence, almost certainly, is part of the gene coding for CK-IV subunits.

Persistence of maternally derived CK-IV/IV isozymes in X. laevis

Oocytes, eggs and early embryonic stages beyond neurulation contain large amounts of CK-IV/IV homo-dimers as the unique CK isozyme. These may represent a stock that was built up during oogenesis, or may be replenished through translation of stored oocytic messengers. Alternatively, it could result from de novo transcription of the CK-IV genes. In order to distinguish between these possibilities, we used Northern blots for the detection of CK-IV messengers, as well as zymograms of the offspring from a mother heterozygous for two CK-IV alleles, CK-IV slow (s) and CK-IV fast (f).

Northern blot analysis of early developmental stages reveals important amounts of maternally derived CK-IV messengers that become undetectable by the time of gastrulation (stage 13); novel CK-IV messengers are again present in equally large amounts at stage 27 (Fig. 2). This profile excludes a possible replenishment of CK-IV isozymes from continued translation of stored messengers, at least after gastrulation.

Zymograms of oocytes and eggs of heterozygous CK-IV/f females show three anodal CK-IV/f bands, namely s/s, s/f and f/f dimers with a staining ratio of 1:2:1 (Wolf and Kobel, 1985). After fertilization with sperm of a homozygous CK-IV f male, 50 percent of the zygotes are expected to be 1/4 CK-IV f and 3/4 CK-IV s. De novo synthesized CK-IV s subunits are visible mainly as heterodimers formed with CK-IV f and with CK-III subunits while homodimers CK-IV f/IV f are less abundant, demonstrating the simultaneous de novo appearance of both CK-III and CK-IV subunits. It is noteworthy that heterodimers apparently are not generated in vivo through reassortment of subunits from preexisting dimeric CK isozymes (see above). Messengers for CK-IV are also detectable in Northern blots at similar stages (Fig. 2). In whole embryos or in isolated head and posterior parts, only very faint CK-II/III bands (estimated to represent less than 1/1000th of total CK activity) and no CK-I can be seen up to stage 47.

The expression of CK-I and CK-II/III isozymes takes place during organogenesis in a tissue-specific manner. The CK-I isozyme, which in the adult is found almost exclusively in eye and gut, is first detected in these organs at stage 45 (Fig. 4). The CKII/III isozyme is the major isozyme of adult skeletal muscle and is also present in appreciable amounts in adult eye and heart. One particular feature of this isozyme is that it is expressed earlier in heart muscle than in other tissues. At stage 42, the beating heart (including surrounding tissues) displays a small amount of CK-II/III, subsequently reaching a level comparable to adult heart at stage 48 (Fig. 4). Activation of CK-II genes in eye and axial skeletal muscle occurs only during metamorphosis. In both organs, CK-II/III activity appears around stage 60 and it is several weeks beyond metamorphosis before the full adult pattern has developed (Fig. 5B). The hindleg as an adult organ develops slowly throughout larval life. Its anlage seems to start with only CK-III/III and CK-IV/IV activity, the latter disappearing rapidly. Between stages 55 and 60, when the leg musculature becomes fully differentiated, CK-III/III heterodimers rise in quantity until, several weeks after metamorphosis, this isozyme contributes the major part of the total CK activity, the remaining being CK-III/III homodimers. Differentiation of new adult muscles in the trunk, as compared to hindlegs, is somewhat

Consequently, maternally derived CK-IV/IV isozymes persist throughout embryogenesis and beyond the stage where tadpoles start to feed.

Developmental profile of CK isozymes in X. laevis

The onset of zygotic CK activity is easy to detect for the CK isozymes not present in the maternally derived stock. In whole embryos (Fig. 5A), the CK-III/III isozyme becomes visible at stage 24 (tailbud). At stage 34 (heart beating), CK-III has also formed heterodimers with CK-IV subunits, suggesting simultaneous zygotic activity of CK-III and CK-IV genes. In order to verify the presumed simultaneous activation of these two CK genes, eggs of a homozygous CK-IV f/IV f female were fertilized with sperm of heterozygous CK-IV f/IV s males giving homo- and heterozygous offspring. Fig. 3B shows zymograms obtained by pooling 50 embryos for each of 10 developmental stages as well as from individual embryos presenting the two possible phenotypes. The allelic content of the samples is expected to be 1/4 CK-IV f and 3/4 CK-IV s. As from individual embryos presenting the two possible phenotypes. The allelic content of the samples is expected to be 1/4 CK-IV f and 3/4 CK-IV s. De novo synthesized CK-IV s subunits are visible mainly as heterodimers formed with CK-IV f and with CK-III subunits while homodimers CK-IV f/IV f are less abundant, demonstrating the simultaneous de novo appearance of both CK-III and CK-IV subunits. It is noteworthy that heterodimers apparently are not generated in vivo through reassortment of subunits from preexisting dimeric CK isozymes (see above). Messengers for CK- IV are also detectable in Northern blots at similar stages (Fig. 2). In whole embryos or in isolated head and posterior parts, only very faint CK-II/III bands (estimated to represent less than 1/1000th of total CK activity) and no CK-I can be seen up to stage 47.
delayed and stretches out over a longer period and beyond metamorphic climax (Ryke in Nieuwkoop and Faber, 1967); this is also clearly reflected by the delayed unfolding of CK-II/III isozymes in the axial musculature. Tail muscles, on the other hand, do not show CK-II/III activity at any metamorphic stage. In other organs, i.e. heart and intestine, there is no difference in the CK phenotype of tadpoles and adults.

Developmental profile in X. borealis

The CK isozymes of X. borealis are presumed to be orthologous to those of X. laevis, by the similarity of the general tissue-specific distribution in both species (Wolff and Kobel, 1985). CK-IV/IV of X. borealis is represented by two anodal bands, a fact which, by analogy to similar double bands of X. gilli (also presumed to be of allotetraploid origin), has been interpreted as conserved activity of duplicated CK-IV genes. The two CK-IV/IV isozymes show different tissue-specific expressions; however, since at least one of the possible CK-III/IV heterodimers comigrates with one of the CK-IV/IV homodimers, the interpretation of zymograms remains uncertain with regard to differential expression of the two CK-IV/IV isozymes. Another difference concerns the major isozyme of skeletal muscle which in X. borealis has not been shown to be a CK-II/III heterodimer as in X. laevis; on the contrary, expression of CK-II/II in oocytes without the simultaneous presence of CK-III/III homodimers suggests that it is a homodimer in X. borealis.
Fig. 5. Overview of the CK isozymes patterns during the development of *X. laevis victorianus* (A–C) and *X. borealis* (D–F). The numbers indicate the different developmental stages according to Nieuwkoop and Faber (1967). (A, D) Pools of 20 whole embryos for each of the 13 developmental stages were homogenized, electrophoresed on cellulose acetate membranes and stained for CK activity. From stage 37, the heads (h) of the embryos were separated from the body (b). (B, C and F) Pattern of CK expression on isolated tissues during metamorphosis. Heart, eye and muscles from the leg, the back and the tail were isolated from individual tadpoles before metamorphosis (stage 54 to 65), at the metamorphic climax (stage 65+) and weeks (w) or months (m) after metamorphosis. (E) Heat treatment during 1 to 2 min at 50°C of whole stage 9 and 37 embryos, and of adult eyes, in order to distinguish between comigrating heat-resistant CK-III/III and heat-sensitive CK-II/IV isozymes of *X. borealis*.

The developmental profile of the various CK iso- zymes of *X. borealis* is rather similar to that of *X. laevis* during embryogenesis and metamorphosis (Fig. 5). The main difference concerns the phenotype of oocytes and early embryos where, in addition to CK-IV/IV, the CK-II/II isozyme is also present in equal amounts. Eggs thus display a 4-banded zymogram: CK-II/II homodimers (see above), CK-II/IV heterodimers and the two CK-IV/IV homodimers. Another interpretation of the zymogram by including CK-III/III activity, whose homodimers would comigrate with the presumed CK-II/IV heterodimers, can easily be discarded by means of the differential heat sensitivity of the various CK iso- zymes. CK-III/III and CK-I isozymes are stable while CK-II/II, CK-II/III and CK-IV/IV are quickly inactivated at 50°C. Fig. 5 E shows zymograms of heat-treated extracts of whole stage 9 and 37 embryos, and of adult eyes. In stage 9 embryos, all CK activity is abolished after 2 min at 50°C, while in stage 37 embryos and adult eye extracts the CK-III/III homodimers stay active. CK-III/III isozymes are thus not present in early embryos but appear before stage 37. It seems reasonable to assume that zygotic activity of CK-III/III and CK-IV/IV is comparable in both species. CK-II/II remains at low levels throughout larval development of *X. borealis* but, as is the case in *X. laevis*, CK-II expression rises dramatically during metamorphosis in parallel to the differentiation of adult musculature in both hindlegs and trunk.

**Discussion**

The expression of CK isozymes in *Xenopus* begins with a large maternally derived stock of CK enzymes that remains active throughout embryogenesis and into larval life. In *X. laevis* maternal CK-IV/IV homodimers are present as the unique CK isozyme at a constant level from oocytes throughout early development until tailbud stage, while its messenger declines rapidly after fertilization and has completely disappeared after gastrulation. Storage of oocytic messen- gers and proteins is a common feature in animal development. Mature oocytes of *Xenopus* contain very large amounts of poly(A)+ RNA (Dawid et al. 1983) and it is often assumed that the biochemical machinery required for early development depends on the translation of the stored mRNAs, until zygotic transcription starts at the midblastula transition, an assumption probably largely inspired by the features of histone metabolism (Woodland, 1980). In the case of CK in *Xenopus*, however, it is the functional homodimeric enzyme that persists and replenishment of its stock
through continued translation appears to be low or absent until zygotic expression begins, much later, around tailbud stage.

Zygotic expression of CK becomes visible only at stage 23/24 (12 to 15 somites) and seems to initiate simultaneously with CK-III/III and CK-IV/IV, while CK-I and CK-II/III remain silent. Northern blots using a cDNA clone specific to the CK-IV/IV isozyme reveal appreciable amounts of novel mRNA at the same period. Since reassociation between zygotic CK-III subunits and maternally derived CK-IV subunits seems not to occur in vivo, the presence of CK-III/IV heterodimers indicates that both genes are active in the same cell type although one cannot assume that all cells express both genes simultaneously. Stage 23 is characterized by the differentiation of myoblasts in the most anterior somites and by the fact that the embryo becomes able to react to external stimulation. Since CK-III/III represents the main CK isozyme of larval muscles, much of the CK-III/III activity seen on zymograms could be contributed by the differentiating somites alone. One can wonder whether the short period of CK-IV/IV activity in somitic musculature results from zygotic transcription or is entirely due to stored CK-IV/IV isozymes. In the eye anlage, on the other hand, CK-III/III appears much later and is still weak at a stage when tadpoles already start to feed (45), whereas the heart displays CK-III/III and CK-IV/IV homo- and heterodimers much earlier so that at stage 45 almost the full adult pattern has unfolded, CK-II/III heterodimers included. Zygotic activation of all four CK genes thus takes place in a precise time sequence and in an organ-specific manner, resulting in a CK phenotype that may represent either the final adult pattern or a temporal larval one as in skeletal musculature and in eyes that lack CK-II/III isozymes as tadpoles.

During metamorphosis, the trunk musculature undergoes profound changes and many new adult muscles are added. Correspondingly, the CK phenotype changes and CK-II/III heterodimers appear. Until several weeks after metamorphosis this isozyme represents, with about 97% of the total CK activity, the CK-III/III activity of adult muscles (Robert and Kobel, 1988). In hindlegs, CK-II/III heterodimers appear much earlier, reflecting the fact that legs directly become able to react to external stimulation. Since CK-III/III represents the main CK isozyme of larvae muscles, much of the CK-III/III activity seen in larval muscles and CK-II/III represents the main isozyme of adult skeletal muscles, both isozymes are also significantly expressed in other tissues like eyes or stomach.

The expression of the various CK genes of Xenopus is not only tissue specific but is also clearly under differential developmental control. It seems reasonable to attribute to each CK isozyme a specialized function brought forth by the structural properties of the isozyme itself. However, the idea (Pickering et al. 1985) that distinctness of biological features may reside at the level of gene regulation rather than in the final gene product, provides an appealing model. One could speculate that divergence in the regulation modalities creates an independence between different CK genes that allows a refined tuning of gene expression even if the encoded polypeptides remain functionally and structurally very similar. The comparable, but in several aspects different, regulation pattern encountered in X. borealis supports this hypothesis.

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