A *Xenopus laevis* creatine kinase isozyme (CK-III/III) expressed preferentially in larval striated muscle: cDNA sequence, developmental expression and subcellular immunolocalization

JACQUES ROBERT*†, BENJAMIN BARANDUN AND HANS RUDOLF KOBEL

Laboratoire de Génétique, Université de Genève, 154 bis route de Malagnou, CH-1224 Chêne-Bougeries, Geneva, Switzerland

Summary

A cDNA containing the nearly complete coding sequence of CK-III subunit of *X. laevis* was isolated, sequenced and further identified by comparing the tissue distribution of CK-III/III isozyme with that of its messenger. Comparison of CK-III deduced amino acid sequence with other CK sequences published reveals its close homology to M-CK subunits. Results using both cDNA probes and monoclonal antibodies specific for CK-III subunits indicate that the appearance and the accumulation of CK-III occur in parallel with myoblast differentiation. Moreover, subcellular immuno-histolocalization shows that CK-III/III isozyme is especially concentrated on larval myofibres at the level of A-bands.

1. Introduction

Creatine kinase (CK) enzymes are phosphotransferases that regulate the cellular ATP metabolism through a creatine–phosphate shuttle (reviewed by Walliman & Eppenberger, 1985). In higher vertebrates, three different types of CK subunits are encoded by separate genes (reviewed by Perriard *et al.* 1987). The B-CK and M-CK subunits combine to three dimeric cytoplasmic isozymes; BB, MB and MM, while the third subunit, the Mt-CK, gives rise to a mitochondria-associated octameric isozyme (Schlegel *et al.* 1988). Characterizations of cDNAs (Babbitt *et al.* 1986; Haas & Strauss, 1990) and genomic clones (Benfield *et al.* 1988) coding for the three types of CK isozymes were reported for several species. The expression of each CK isozyme is tissue-specific and developmentally regulated (Perriard *et al.* 1987). Embryos first express BB-CK, which occurs also in most adult tissues. The differentiation of skeletal muscle and myocardium is accompanied by the induction of M-CK, which becomes the major CK isoform of mature muscle cells.

In contrast to several amphibian species that show a comparable CK isozyme repertoire (Eppenberger *et al.* 1967; Fisher *et al.* 1980; Klemann & Pfohl, 1982) the more complex CK isozyme system of *Xenopus laevis* resembles that of certain teleost fish (Fisher & Whitt, 1978; Whitt, 1981). It involves at least four separate CK genes, all of which are differentially regulated (Wolff & Kobel, 1985; Robert *et al.* 1990). CK-I isozyme is restricted to eye and stomach. CK-IV/IV isozyme displays a generalized tissue distribution in both embryonic and adult tissues comparable to that of the B-CK of mammals and chicken or to the CK-C of fishes. On the other hand, larval muscles contain CK–III/III homodimers as the only CK enzyme, but at metamorphosis these are replaced by CK-II/III heterodimer. CK-II subunits can form neither homodimers nor heterodimers with CK-IV subunits, whereas CK-IV and CK-III do combine to heterodimers *in vivo*, and *in vitro* also with rabbit M-CK subunits (Bürki, 1985; Robert & Kobel, 1988).

Since most *Xenopus* species are of allopolyploid origin (reviewed by Kobel & Du Pasquier, 1986), the uncommon complexity of the CK system of *X. laevis* may be reminiscent of the tetraploid origin of this species.

2. Materials and methods

(i) *Animals*

Xenopus laevis laevis were imported from Cape Town, South Africa or laboratory raised. Developmental stages are according to Nieuwkoop & Faber (1967).

(ii) *Isolation and characterization of cDNA clones*

An *X. laevis* cDNA library from stage 40 larvae was screened at high stringency with a probe derived from

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* Present address: Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland
† To whom correspondence should be addressed.

a genomic DNA fragment that was known to contain sequences specific for the CK-III subunit (Robert *et al.* 1990). The various cDNA and genomic DNA fragments were subcloned in Bluescript (Stratagene) plasmid vectors. Sequence analysis was performed on denatured double-stranded recombinant plasmid DNA (Zhang *et al.* 1988) by the standard dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase enzyme (USB).

(iii) *RNA isolation and analysis*

RNA extraction and Northern blot analysis were performed as described (Robert *et al.* 1990). Total cellular RNA sample (10 mg), isolated from pools of 200 embryos at different stages, and from different organs of adult *X. laevis* by the Li/urea procedure (Aufray & Rougeon, 1980) were denatured with glyoxal (Thomas, 1983), resolved on 1 % agarose gel and blotted. Hybridization was performed for 48 h at 42 °C with ^{32}P-labelled genomic or cDNA fragments. The sizes of mRNAs were determined using RNA markers (Boehringer). We verified that each lane of the blots contains equal amounts of RNA by reprobing the membranes with an *X. laevis* rDNA probe (G. Spohr, personal gift).

(iv) *Immunohistology*

Tadpoles (stages 40–50) were fixed for 6 h at room temperature in alcoholic Bouin's fixative (Humason, 1979), washed in 94 % ethanol, dehydrated and embedded in paraffin. Sections of 5 μm were mounted on 0·1 % poly-L-lysine-coated glass slides, deparaffinized in xylene, rehydrated and pre-incubated 1 h in 0·1 % NP-40, 10 % goat serum, 1 % BSA in TBS (1 % NaCl, 50 mM Tris; pH 7·5). Sections were then immunostained by the ABC method (Hsu *et al.* 1981) as follows: (1) incubated overnight at 4 °C with mAbs diluted with 1 % goat serum in TBS, (2) rinsed with 0·05 % Tween-20 in TBS, (3) incubated 1 h with biotinylated goat anti-mouse Ig (Vector, USA), which was pre-adsorbed with red cells of *X. laevis* and diluted 1:200 with 1 % goat serum, 1 % *Xenopus* serum in TBS, (4) rinsed again with 0·05 % Tween-20 in TBS, (5) treated 30 min with 0·6 % H$_2$O$_2$ in methanol, (6) rinsed in TBS, (7) incubated 30 min in AB complex (Vectastain ABC kit, Vector), (8) rinsed in TBS, (9) incubated 10 min with 0·05 % Diaminobenzidine tetrahydrochloride, H$_2$O$_2$ 0·01 % made up in 0·1 M Tris (pH 7·2), and (10) counterstained with haematoxylin. Controls were currently processed by using non-specific hybridoma supernatants, or by pre-adsorbing the mAb supernatants with purified CK-III/III isozymes, before application on tissue sections.

3. Results

(i) *Isolation and characterization of a CK-III cDNA clone*

Among several clones previously isolated from an *X. laevis* genomic library (Robert *et al.* 1990), one of them, pGX50, was shown by further characterization, including partial sequencing as well as Southern and Northern blot analysis, to contain the CK-III gene. We used a *Hind* III/*Eco*R 1 fragment of this clone as probe to screen at high stringency an *X. laevis* cDNA library from stage 40 larvae (kindly provided by Dr G. Spohr, Geneva). Five clones were isolated and characterized by restriction analysis, one of which, pXCK4, carrying the largest insert, was completely sequenced. When compared to published CK sequences of mammals and chickens, pXCK4 starts in the coding region 180 nucleotides downstream from the ATG triplet and stops within the 3′ untranslated region.

Alignment of the pXCK4 sequence at the amino acid level with those of *X. laevis* CK-IV, chicken B- and M-CK and of *Torpedo marmorata* CK (Fig. 1) reveals a high degree of similarity, without any insertion or deletion. The C-terminal sequence Pro-Ala-Gln-Lys, common to all known CK sequences (Babbitt *et al.* 1986), as well as the Cys residues of the active site are equally conserved in both CK sequences of *X. laevis*. Trp and Cys residues are thought to be spatially near the active site of CK enzymes (Kenyon & Reed, 1983). The four Trp residues, being at invariable positions in published sequences, are also conserved for both *X. laevis* CK sequences. In addition to the cysteine-283 of the active site, two other Cys residues of the XCK4 cDNA also have positions identical to chicken and mammalian sequences. Preliminary sequencing of genomic fragments (data not shown) suggests evolutionary conservation among CK genes of different species, since an intron at position 910 in both *X. laevis* CK-III and CK-IV coding sequences is found at exactly the same location in the rat B-CK and M-CK genes (Benfield *et al.* 1988). Moreover, the coding sequence of the CK-III-specific genomic fragment, pGX35, is strictly identical with that of pXCK4 cDNA.

The degree of similarity between XCK4 deduced amino acid sequence with respectively those of M or B type chicken CK subunits (Table 1) indicates a closer similarity with the M type, 89 % against 83 % with B-CK. Homology between CK-III cDNA and M-CK of mammals such as rabbit and rat, as well as dog and man, remains between 89 and 90 %. Among the 34 positions that could consistently differentiate between the M and B forms in higher vertebrates (Babbitt *et al.* 1986; starred in Fig. 1), CK-III is identical at 27 to the M form, and at 4 to the B form, while 3 are unrelated. For CK-IV, the numbers are
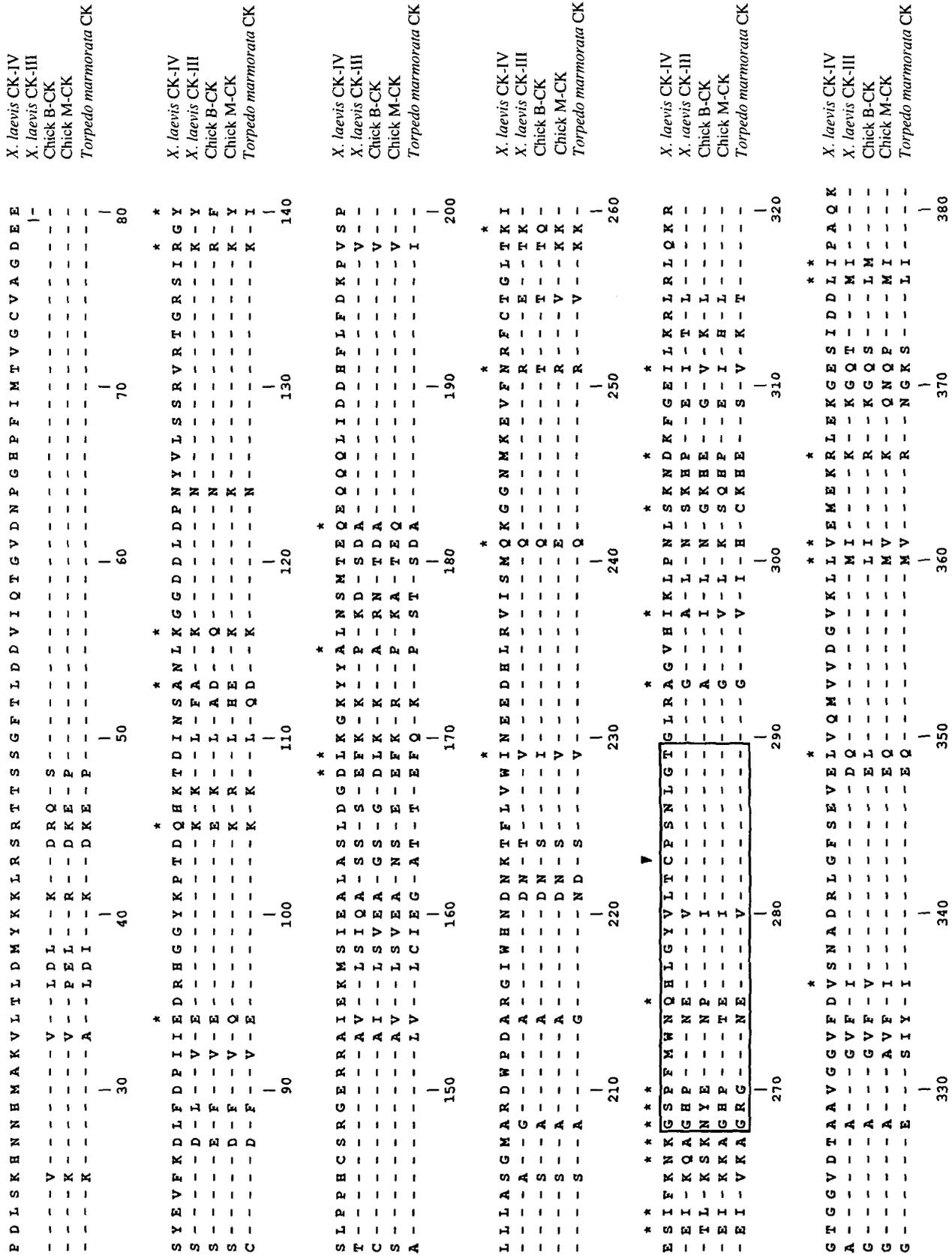
Fig. 1. Alignment at the amino acid level of *X. laevis* cDNA clones pXCK1 (CK-IV, Robert *et al.* 1990) and pXCK4 (CK-III), with chicken B and M-CK isoforms and *Torpedo marmorata* CK (Babbitt *et al.* 1986). Numbering is from the methionine at position 1 of chicken B-CK subunit. * indicates residues with a consistent difference between the M and B subunit for the higher vertebrates. The active site (residues 268–292), with the cysteine-283 (marked with an arrow), is framed.

Table 1. *Sequence identities at the amino acid level between* X. laevis *CK-III and CK-IV cDNA sequences and those of other vertebrates*

		Homology (%) X. laevis	
		CK-III	CK-IV
X. laevis	CK-III	—	85
Torpedo	—	84	82
Chicken	M-CK	89	84
Chicken	B-CK	83	89
Rabbit	M-CK	89	85
Rabbit	B-CK	79	84
Rat	M-CK	90	85
Rat	B-CK	80	84

respectively 11, 15 and 8. The sequence concordance with the *Tornado marmorata* CK is weaker.

(ii) *Identification of CK-III transcript by Northern blotting*

Northern blot analysis (Fig. 2) shows that both XCK41 cDNA and the pX35 genomic fragment recognize a 1·7 kb RNA present only in adult skeletal muscle and heart, or in whole larvae older than stage 27. On the other hand, tissues such as ovary or kidney, which do not contain any CK-III/III or CK-II/III activity, show no signal. The size of CK-III transcripts, 1·7 kb, is slightly bigger than that of CK-IV subunits (1·6 kb, Robert *et al.* 1990). Appearance of zygotic CK-III transcripts occurs simultaneously with that of CK-IV, at stage 27. This distribution agrees with the tissue distribution of the CK-III subunit, as revealed by specific mAbs and zymogram analysis. The CK-III is expressed as homodimers CK-III/III in larval skeletal muscles before metamorphosis and as heterodimers with CK-II subunits in adult musculature (Robert & Kobel, 1988).

(iii) *Subcellular immunohistolocalization*

JRM4 monoclonal antibody was shown to be specific for both denatured CK-III subunit and native CK-III/III homodimers, but does not cross-react to heterodimeric CK-II/III (Robert *et al.* 1991). On immunohistological sections of tadpoles before metamorphosis, it binds exclusively to skeletal muscles and to muscle fibres of the heart atrium. At higher magnification, the positive anti-CK-III staining spreads all over the cytoplasm, but not uniformly. Transverse sections of myotomal muscle, for example (Fig. 3A, C), reveal that CK-III antigens are principally accumulated in myofibres. Plasma membrane, as well as sarcoplasm at the periphery and between fibre bundles, is much less stained. Myofibres on longitudinal sections (Fig. 3B, D) show a characteristic
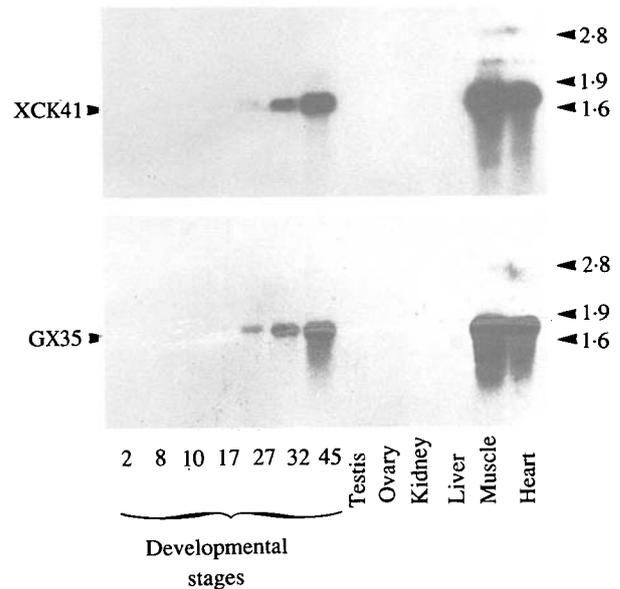
Fig. 2. Northern blot hybridization of pX41, a 500 bp Pst fragment from pXCK4 (CK-III) cDNA and of the genomic GX35 fragment, to total RNA from various tissues and various developmental stages of *X. laevis*. The 10 mg RNA samples were denatured with glyoxal, resolved on 1 % agarose gel and transferred to nitrocellulose membranes. Hybridization was performed for 48 h at 42 °C with the P^{32}-labelled fragments. The sizes of mRNAs were calibrated using RNA marker (Boehringer). We verified that each lane of the blot contains equal amounts of RNA by re-probing the membrane with an *X. laevis* rDNA probe.

striated staining pattern. Observation by phase contrast, in order to reveal the Z-line that separates each sarcomere, indicates that CK-III antigens are localized at the position of A-bands. The same specific pattern is found on muscle myofibres from the heart atrium.

After metamorphosis, this antibody no longer reveals CK-III/III isozymes in skeletal muscle (Robert *et al.* 1991), in contrast to zymograms of adult muscle extracts, where this isozyme is represented as a minor band, and to Northern blots demonstrating large amounts of corresponding mRNAs (Fig. 2).

4. Discussion

The differential expression of CK isozymes during muscle development of *X. laevis* is of particular interest, since two successive transitions take place. After neurulation, larval muscle differentiation is accompanied by the specific expression of CK-III/III isozymes. Later on, activation of the CK-II gene at metamorphosis results in the almost complete replacement of CK-III/III homodimers by CK-II/III heterodimers. In adult skeletal muscle, CK-II/III isozymes represent more than 95 % of the total CK activity, the remainder being due to CK-III/III (Robert & Kobel, 1988). One may, however, question if CK-III/III homodimers exist at all in adult muscle, since monoclonal antibodies specific for CK-III/III
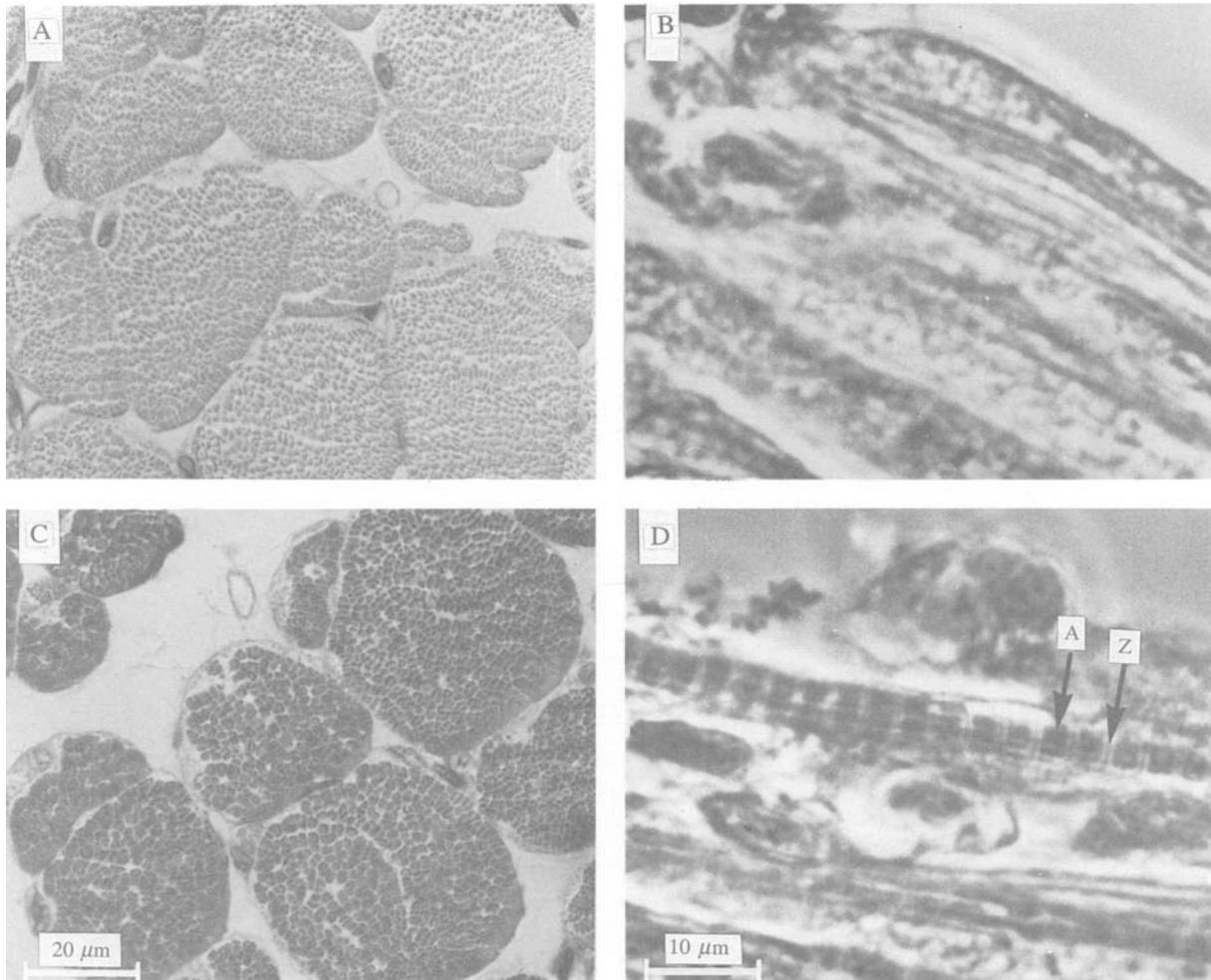
Fig. 3. Immunolocalization of CK-III antigens in transversal section of myotomal muscle (A, C) and in longitudinal section of interhyoideus muscle (B, D), of *X. laevis* larvae at stage 42 by the ABC staining technique. (A, B) Controls with non-specific hybridoma supernatant; (C, D) anti-CK-III (JRM4) mAb. Myofibres in longitudinal sections (B, D) are visualized by phase contrast; CK-III antigens are concentrated between Z-lines at the level of A-bands.

do not detect this isozyme in immunohistological staining of adult muscle. It is possible that the apparent presence of CK-III/III in extracts is due to reassociation of subunits from CK-II/III hetero-dimers, that easily occurs *in vitro* through storage, thawing–freezing or reversible dissociation by urea (Robert & Kobel, 1988).

The CK-III locus was first identified through its developmental and tissue-specific expression (Wolff & Kobel, 1985). Isolation of genomic and cDNA clones, as well as monoclonal antibody specific for CK-III subunits, permitted further characterization of the corresponding CK-III gene and its expression. The deduced amino acid sequence of CK-III cDNA is very similar to that of M-CK of mammals. Hence the CK-III locus is most likely analogous to the M-CK locus of mammals and chicken, and more generally to the CK-A locus of the chordate branch of animals (Fisher *et al.* 1980). Moreover, immunohistological studies reveal that CK-III antigens are concentrated speci-fically on myofibres, especially at the site of A-bands.

This subcellular localization is comparable to that of M-CK in dog myocard (Otsu *et al.* 1989). A more detailed study by electron microscopy would ascertain if a fraction of CK-III/III is specifically associated with the M-band (Walliman & Eppenberger, 1985).

Among the four CK loci of *X. laevis*, analogy to M-CK and B-CK of other vertebrates has now been established for CK-III and CK-IV, respectively. Of the remaining loci, little is known about CK-I except that it has a very restricted tissue expression resembling in some ways that of mitochondrial CKs. CK-II, on the other hand, shows a peptide pattern (Robert & Kobel, 1988) and a tissue distribution (Robert *et al.* 1990) much like that of CK-III. This suggests that these two loci resulted from duplication subsequent to allotetraploidization, though the question can only be settled once the sequence of CK-II becomes available. Other tetraploid Xenopus, i.e. *X. borealis* and *X. gilli*, show a similar transition of muscle CKs at meta-morphosis. These species seem also to have retained duplicated CK-IV loci (Wolff & Kobel, 1985) in

contrast to *X. laevis*, where we could find no indication for duplicated B-CK genes.

It is conceivable that duplicated M-CK loci have been retained because they acquired different tissue-specific and developmental regulation. The evolutionary use of duplicated genes to modulate their expression rather than to achieve a structural specialization of their products is illustrated by variation of the tissue-specific and ontogenic CK isozyme repertoire from one species to another. In *X. borealis*, for example, the CK-II gene is expressed during oogenesis and embryogenesis, while in *X. laevis* the first activity of this gene is discernible in hearts of developmental stage 45, but otherwise is not activated before metamorphosis (Roberts *et al.* 1990).

We wish to thank Professor H. Gloor for reading the manuscript and making helpful suggestions.

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