Calnexin: its molecular cloning and expression in the liver of the frog, *Rana rugosa*

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Abstract A 2.2-kb cDNA clone encoding the endoplasmic reticulum (ER) resident protein, calnexin (CLX), was isolated from the frog *Rana rugosa* liver cDNA library and sequenced. The sequence encoded 622 amino acids and was 77% similar to mammalian CLXs and 56% to mouse CLX-t. In the phylogenetic tree, mouse CLX-t was clearly diverged from other CLXs including frog. The amino acid sequence of CLX showed regions similar to those in frog calreticulin (CLT), although CLX, but not CLT, contained a histidine-rich region at the NH_2-terminus. CLX gene expression was observed only in the liver among various tissues examined. Additionally, its expression was strong in the liver in 2-month post-metamorphosis frogs, but very weak in adults. The results suggest that the CLX gene is expressed in a tissue- and stage-dependent manner in the frog, *R. rugosa*, and that CLX is widely distributed in eukaryotic organisms.

Key words: Frog; Calnexin; cDNA cloning; Expression in liver cells

1. Introduction

In recent years, calnexin (CLX), a Ca^{2+}-binding protein, has attracted considerable attention. CLX represents a new type of molecular chaperone [1]. CLX is known to be part of the endoplasmic reticulum (ER) quality control machinery, binding and folding intermediates through their oligosaccharide moieties until these substrates achieve proper folding or until misfold proteins are degraded [2,3]. In *Saccharomyces pombe*, CLX is essential for viability [4]. It is also known that the intraluminal domain of CLX shares considerable sequence similarity with another ER Ca^{2+}-binding protein, calreticulin (CLT), including three KPEDWD repeats in the central proline-rich domain [5,6]. However, the current knowledge about CLX and CLT is limited with regard to its actual cellular function, structural features, and regulation. CLX is suspected to be widely distributed throughout species, because its cDNA was isolated and sequenced from mammals, microorganisms and plants. It is, however, very important to clone the CLX cDNA from animals other than mammals. By this, the general structure of CLX would provide evidence of whether it has been conserved through evolution.

Here we report the isolation of the complete CLX cDNA, and describe its gene expression in the liver of the frog, *Rana rugosa*.

2. Materials and methods

2.1. Isolation and sequencing of cDNA clones

A λ gt10 cDNA library derived from frog liver mRNA was screened with rat CLT cDNA as probe according to Maniatis et al. [7]. In brief, a λ gt10 phage was adsorbed to MRA bacteria and grown on NZYM plates for 7 h at 37°C. Approximately 5×10^5 phages were screened on 15 nitrocellulose filters (Advantec). The filters were prehybridized for 4 h at 60°C in a prehybridization solution containing 5×Denhardt's solution, 0.5% SDS, 6×SSC and 100 μg/ml of salmon sperm DNA. The filters were hybridized for 16 h at 60°C in a fresh hybridization solution with 1×10^6 cpm/ml of probe, washed twice for 15 min at 22°C with 2×SSC and 0.1% SDS, and washed once for 15 min at 60°C with 0.2×SSC and 0.1% SDS. Then the filters were exposed to Fuji RX X-ray films (Fuji) overnight at −80°C. Clones were plaque-purified and subcloned into pUC19 vector before sequencing by the dideoxy chain termination method [8].

2.2. Northern-blot analysis

Total RNA was isolated from unfertilized eggs, larvae, tadpoles and different tissues of young and adult frogs. RNA was electrophoresed on a 1.0% agarose gel and electrophoretically transferred to nitrocellulose membranes (Advantec). The RNA was then hybridized with ^{32}P-labeled 430-bp *Eco*RI/*Mva*I fragments of CLX cDNA as probes, washed at 65°C in 0.2×SSC and 0.1% SDS, dried and then exposed to Fuji RX X-ray films. Tadpoles were staged according to Shumway [9], and Taylor and Kollros [10].

2.3. Sequence analysis

The phylogenetic tree was constructed by the UPGMA method [11] using the program included with PHYLIP [12].

3. Results

3.1. Isolation of cDNA clones encoding CLX

From a λ gt10 expression library constructed from an adult frog liver mRNA, a 2.2-kb clone was obtained. The insert cDNA was subcloned into pUC19 and sequenced, Fig. 1 shows the nucleotide and deduced amino acid sequences from the 2154 bp cDNA clone. Although we have neither purified CLX from frog livers nor determined the NH_2-terminal amino acid sequence, we believe the first ATG, beginning with nucleotide 38, would be the initiating methionine (Met). Reasons are as follows: (a) searching the GenBank data base indicated that the amino acid sequence shares 76.7% identity with dog CLX [5], (b) dog CLX begins with a 20-residue signal sequence and the first Met is followed by positively charged and hydrophobic residues [5], and (c) processing sites of signal sequences are generally longer than 15 residues [13]. The first 20 residues are probably a signal peptide, indicating that the cDNA would encode a protein of 602 amino acids with a calculated molecular mass of 68 509 Da. This sequence

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The nucleotide sequence data of CLT and CLX reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession numbers D78589 and D78590, respectively.

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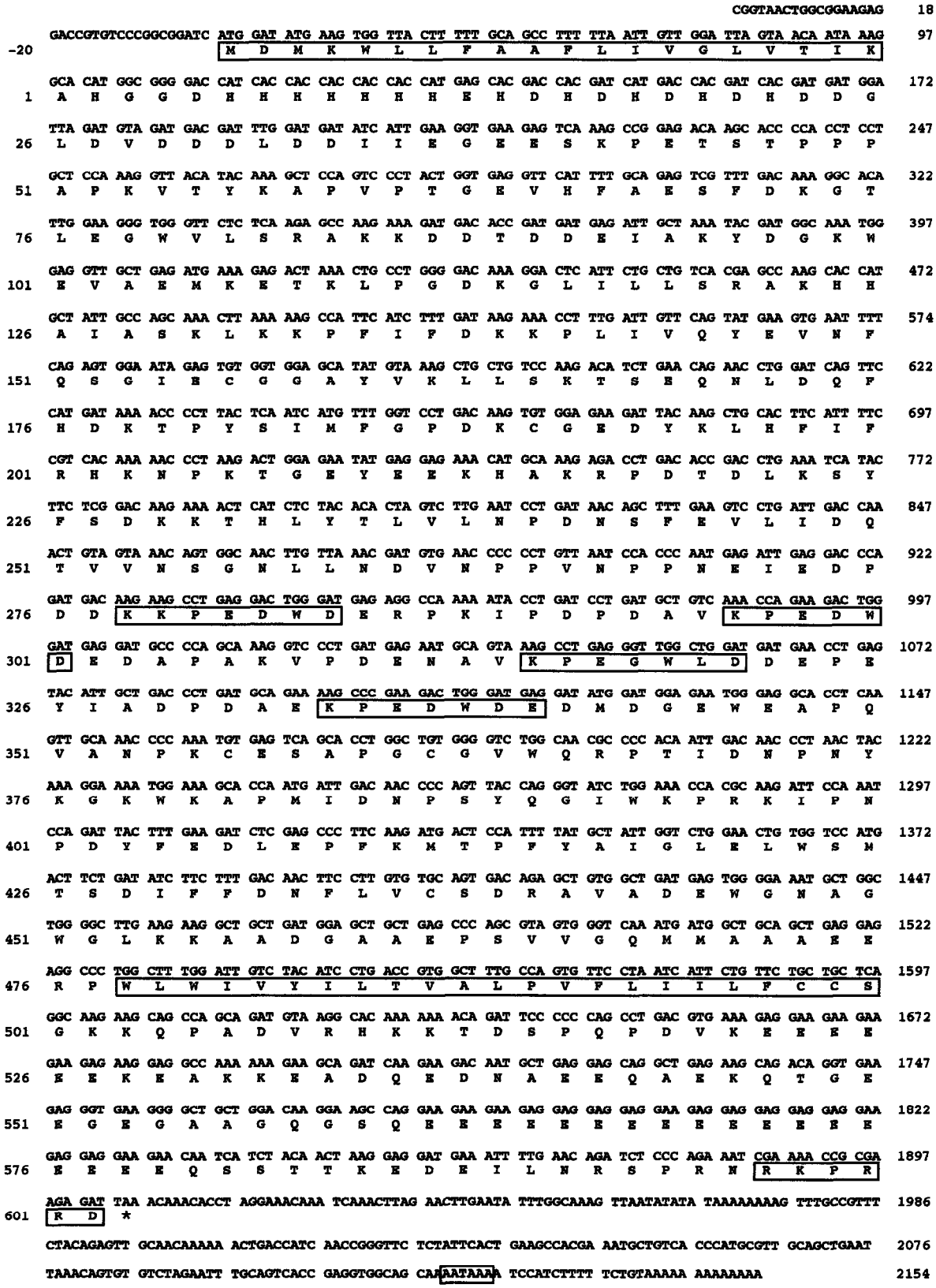

Fig. 1. Nucleotide and predicted amino acid sequence of frog liver CLX. The amino acid sequence deduced from the nucleotide sequence of CLX is shown. A signal peptide of CLX was predicted by the hydrophobicity plots [14]. Amino acid residues are numbered negatively within the signal sequence with amino acid residue 1 corresponding to the first residue of the mature processed protein. The signal peptide, four repeating motifs, transmembrane region and polyadenylation signal are boxed.

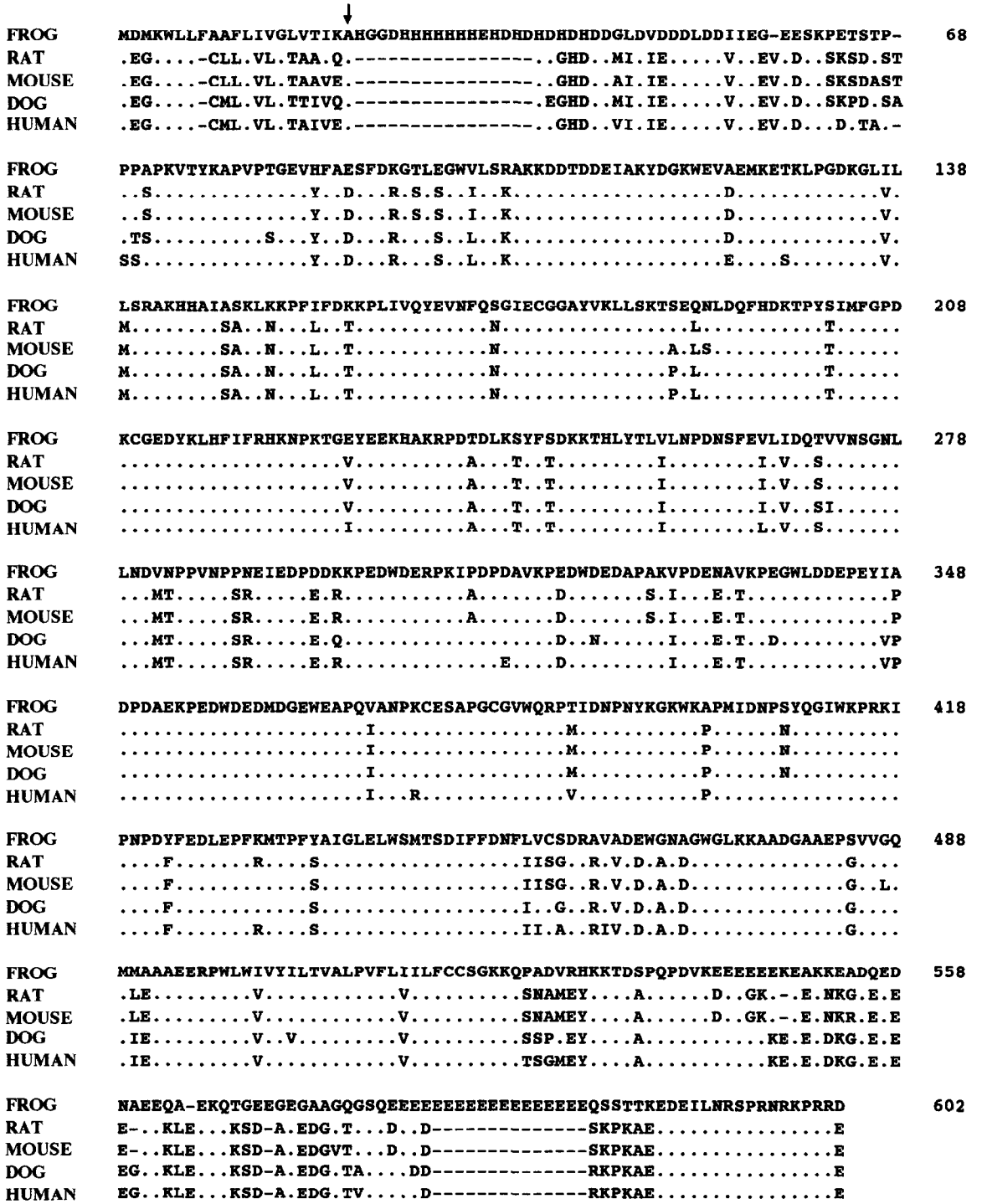

Fig. 2. Comparison of the amino acid sequence of different CLXs. The amino acid sequences of CLXs are taken from [15] for rat and mouse, [5] for dog and [6] for human CLXs. Dots represent perfect matches and dashes represent gaps. The arrow indicates a putative processing site of a signal sequence.

predicts a large intraluminal domain of 477 amino acids, a transmembrane segment of 22 amino acids at positions 461–482 as seen in dog [5] and human [6] and a cytoplasmic carboxyl-terminal tail of 103 amino acids. CLX contains 13 histidines within the first 22 amino acids at the NH_2-terminus, which is unusual among CLXs.

Next, the comparison of amino acid sequences of different CLXs was made. As shown in Fig. 2, deduced amino acid sequences of other known CLX cDNAs were compared, and as a result all sequences were found to be extremely similar (Fig. 2). This indicates that CLX is highly conserved.

Fig. 3 shows sequence similarities between frog CLX and an intraluminal Ca^{2+}-binding protein, calreticulin (CLT). The overall amino acid sequence of frog CLX revealed only 39.6% identity with frog CLT. In the amino acid sequence of CLX, however, there are four internal repeated sequences, designated motif 1 (KIPDPDAXKPEDWDED; residues 271–286, 288–303, 307–322 and 326–341) and motif 2

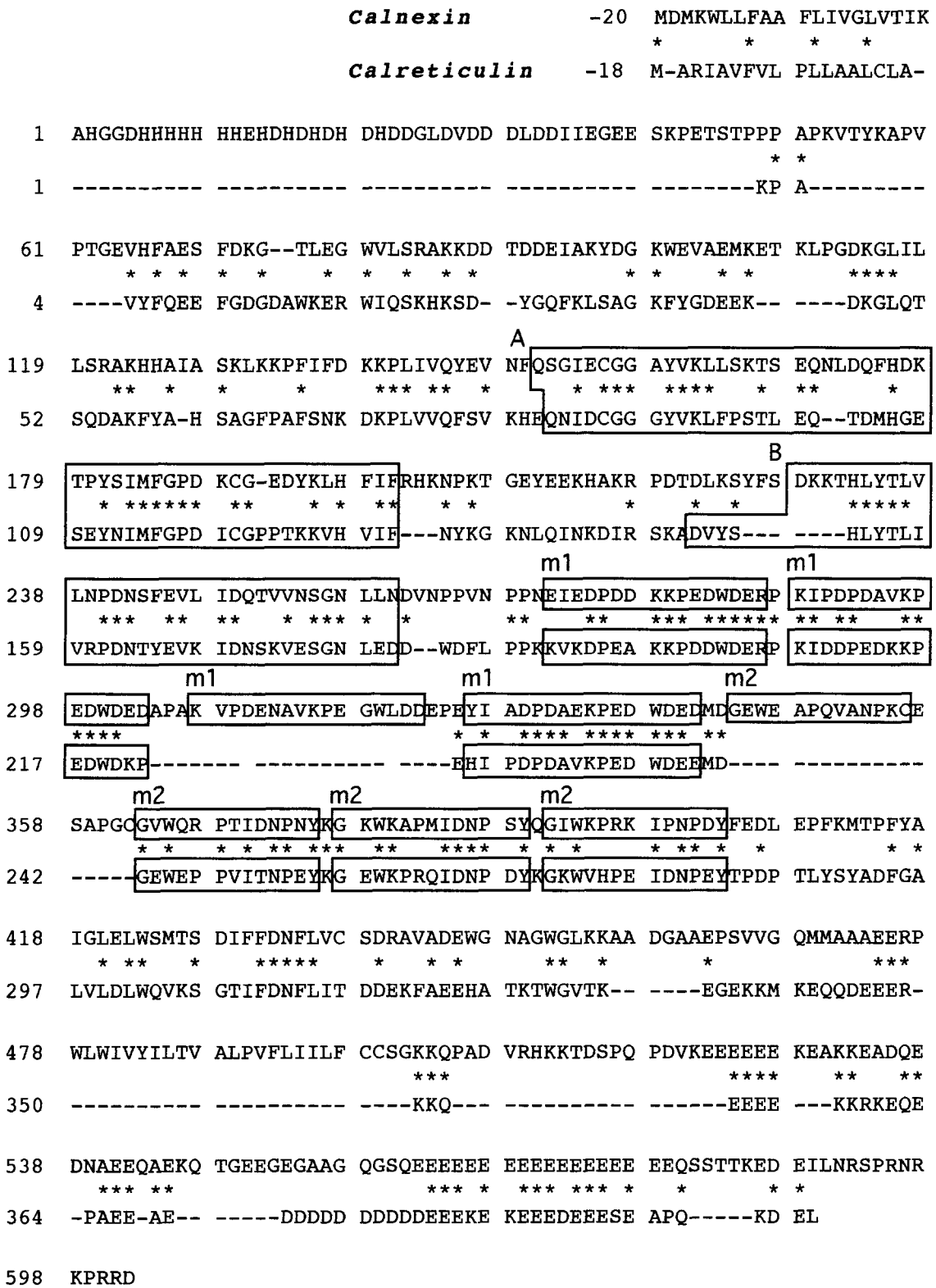

Fig. 3. Sequence similarities between frog CLX and CLT. Signal peptides of CLX and CLT were predicted by the hydrophobicity plots [14]. The CLX cDNA encodes 602 amino acids, while the CLT cDNA encoded 419 amino acids including an 18-residue signal sequence. Amino acid residues are numbered negatively within the signal sequence with amino acid residue 1 corresponding to the first residue of the mature processed protein. Two regions A and B with homology between CLX and CLT (boxed), and four repeating internal sequences in CLX and three in CLT, designated motif 1 (boxed) and motif 2 (boxed), are shown. Identical sequences are indicated with stars. Gaps introduced to optimize alignments are shown with dots.

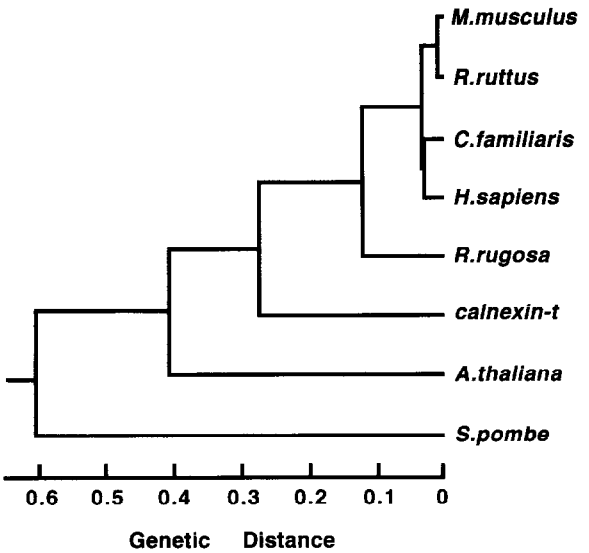

Fig. 4. Evolutionary distance of CLT from different sources at the amino acid level. The dendrogram of CLTs from various sources, based on the amino acid sequence, was generated by the UPGMA method. The GenBank accession numbers of CLX cDNA sequences are: mouse, L18888 [15]; rat, L18889 [15]; dog, X53616 [5]; human, L10284 [6]; mouse CLX-t, U08373 [18]; *A. thaliana*, Z18242 [20]; *S. pombe*, U13389 [4].

(GXWXXPXIXNPXY; residues 344–356, 363–375, 377–389 and 391–403). The former region is acidic (D and E contents, 39%), but the latter is not. Motifs 1 and 2 were also found in CLT, and were repeated three times in tandem. There were also two regions showing sequence similarity between CLX and CLT. Region A was located at residues 153–202 in CLX with 52% identity to CLT, and region B at residues 228–261 with 58% identity. The C-terminus of frog CLX has poly E (18 residues), while that of frog CLT has poly D (10 residues). In contrast, mammalian CLX and CLT have neither poly D nor poly E in the C-region [5]. Frog CLX has the motif RKPRRD at the C-terminus. Lysine (K) at the −3 position of motif KXKXX or RXKXX has been shown to be very important for retaining proteins in the ER [16,17]. Frog CLX has Arg (R) at the −3 position. It should therefore be examined whether the RKPRRD motif at the C-terminus in frog CLX can be an ER retention signal.

3.2. Phylogenetic tree

The frog CLX amino acid sequence shares 76.6% similarity with mouse CLX, but only 56.2% with mouse CLX-t, although the open reading frame of mouse CLX had 60% similarity with mouse CLX-t [18,19]. Thus, the phylogenetic tree was constructed from the pairwise matrix of genetic distance of frog and mouse CLXs and mouse CLX-t at the amino acid level with the UPGMA method. In the tree, plants were clearly separated from vertebrates (Fig. 4). The evolutionary distance between mouse CLX and CLX-t was 0.2775, while that between mouse and frog CLXs was 0.1553. Mouse CLX-t was clearly diverged from frog, human, dog, rat and mouse CLXs (Fig. 4).

3.3. RNA Northern blot analysis

RNA was extracted from whole larvae, liver of tadpoles and various tissues of the frog, *R. rugosa*. Then the RNA was electrophoresed, blotted and probed to determine the expression of the CLX gene during development. When the 5′-cDNA of CLX was used as probe, a single message of 2.2 kb was detected (Fig. 5). In adult frogs, the CLX gene was weakly expressed in the liver, but not in other tissues examined (Fig. 5X). The expression of the CLX gene was also examined in larvae, and the liver of tadpoles and frogs. CLT gene expression was not observed in eggs (stage 1) and larvae at stages 19–25. However, its expression was fairly strong in the liver of 2-month post-metamorphosis frogs, but very weak in adult frogs (Fig. 5Y).

4. Discussion

CLX has been expected to be ubiquitous among eukaryotic organism and conserved through evolution, since CLX cDNAs have been cloned from human [15], mouse [15], dog [5], plants [20] and microorganisms [4]. However, this is not enough to demonstrate that CLX occurs widely in eukaryotic organisms. In this study, we report the isolation of a 2.2-kb cDNA clone encoding the frog, *R. rugosa* CLX, which is the first report concerning the molecular cloning and sequencing of CLX in animals other than mammals. The primary structure, deduced from the cDNA sequence of CLX in the frog *R. rugosa*, would therefore provide the common features for its molecular structure and function. Dog and human CLXs share 94% amino acid sequence identity [5,15]. A CLX homologue isolated from the plant *Arabidopsis thaliana* has been found to be 48% identical with dog CLX [20]. The frog CLX is 77% identical with mammalian CLXs such as human, dog and mouse. This is reasonable, because amphibians are the stock from which birds, reptiles and mammals evolved. The proline-rich region of mammalian CLX molecules is conserved to a high degree, while the first and last 100 residues excluding the last 15 residues at the C-terminus are highly diverged. This is also true in CLXs of *A. thaliana* [20] and *S. pombe* [4]. Based on these observations, it seems likely that

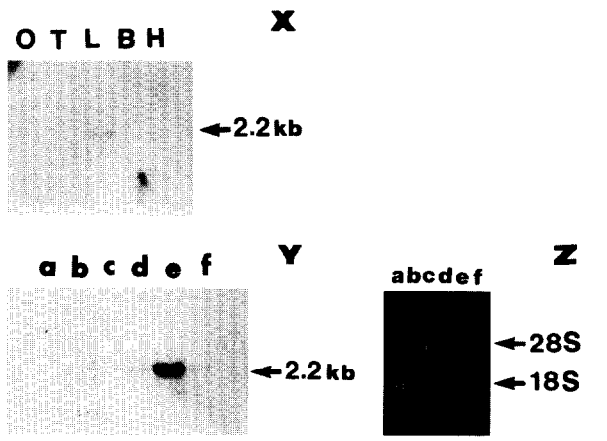

Fig. 5. Expression of CLX mRNA. Total RNA was isolated from the ovary (O), testis (T), liver (L), brain (B) and heart (H) of adult frogs as described in Section 2. Total RNA was also extracted from eggs at stage 1 (a), whole larvae at stages 19–20 (b) and 24–25 (c), and from liver of tadpoles at stages IX–XIII (d), 2-month post-metamorphosis frogs (e) and adult frogs (f). 20 μg of RNA was separated by electrophoresis in 1.0% agarose gels, and blotted to nitrocellulose membranes. Total RNA stained with ethidium bromide is shown in the panel Z. The position of 28S and 18S ribosomal subunits is indicated with arrows. The size of the mRNA hybridizing to the CLX cDNA was estimated to be 2.2 kb.

CLX is a widely distributed protein in eukaryotic organisms and probably serves a key role in cellular functions such as Ca^{2+} storage and a molecular chaperone, aiding the transfer of cell surface molecules in transit from the ER to the outer cellular membrane.

A mouse CLX homologue called CLX-t, which is 60% similar to mouse CLX, has been cloned and sequenced [18,19]. The amino acid sequence of CLX-t is divided into three regions, i.e. N- (amino acids 1–300), P- (301–450), and C- (451–611) regions. The P-region was found to be very similar to that of CLX [18]. Like CLX, there are two motifs in the P-region of CLX-t which are repeated four times in tandem. Based on the similarity of amino acid sequences between CLX and CLT, Ohsako et al. [18] proposed that mouse CLX-t is a CLX variant. However, frog CLX and mouse CLX-t share only 56.2% identity. In the phylogenetic tree, mouse CLX-t is clearly diverged at the amino acid level from CLXs of vertebrates. It might be necessary to reconsider whether mouse CLX-t is a member of the CLX family.

Frog CLX and CLT have the KPEDWD motif in the P-region. This is the highest conserved region when CLX molecules are compared. The P-domain in CLT contains a site for high-affinity Ca^{2+} binding [21]. Mammalian CLXs also contain the P-domain which binds Ca^{2+} with high affinity [15]. We have no evidence at present for abilities of Ca^{2+} binding in the P-domain of frog CLX and CLT. The region at residues 279–339 in frog CLX is 88.7% similar to human CLX, and is also suspected to bind Ca^{2+}. However, we need to prepare fusion proteins with subdomains of the frog CLX molecule in order to analyze abilities of Ca^{2+} binding. No other tissue except liver showed CLX expression. CLX gene expression is probably limited to tissue and developmental stages. This might be one of the reasons why the molecular cloning and sequencing of CLX has not been successful in animals other than mammals. The present study does not provide any evidence concerning the role(s) of CLX in the liver of the frog, *R. rugosa*. Nevertheless, it is of extreme interest that the CLX gene is expressed in the liver of frogs after metamorphosis. Further investigation will answer questions with regard to the role(s) of CLX in the liver.

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