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Molecular Cloning of cDNA for the ζ Isoform of the 14-3-3 Protein: Homologous Sequences in the 3'-Untranslated Region of Frog and Human ζ Isoforms

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ABSTRACT—14-3-3 proteins constitute a family of well-conserved eukaryotic proteins that possess diverse biochemical activities such as regulation of gene transcription, cell proliferation and activation of protein kinase C. At least 7 subtypes (α to θ) of 14-3-3 protein are known, but the ζ subtype of this protein has been cloned only in mammals. We cloned the ζ subtype of 14-3-3 protein (14-3-3ζ) from the frog, *Rana rugosa*. The sequence encoded 245 amino acids that share 92% identity with rat and bovine 14-3-3ζs, and 92% with human phospholipase A2 (PLA2; 14-3-3ζ). Northern blot analysis revealed a single band of about 1.8 kb in tadpoles at stage 25. The 14-3-3ζ mRNA level was high in the brain, lung, spleen and kidney, and low in the heart and testis, as opposed to the mRNA level, which was only faintly detected in the liver, pancreas, ovary and muscle. Furthermore, high similarity in the 3'-untranslated region (3'-UTR) was observed between frog and human 14-3-3ζ cDNA. The results suggest that 14-3-3ζ is highly conserved throughout eukaryotic evolution, and that the homologous sequence in the 3'-UTR of 14-3-3ζ cDNA may be conserved in frogs and humans.

INTRODUCTION

The 14-3-3 protein was originally isolated by systematic analysis of brain protein as a series of acidic proteins with molecular weights of around 30 kDa (Moore and Perez, 1967); it represents a family of dimers consisting of seven isoforms (Ichikawa *et al.*, 1988). This protein was first identified as functioning in catecholamine and serotonin biosynthesis (Ichikawa *et al.*, 1988). However, members of the 14-3-3 protein family were found to be distributed widely in eukaryotes, including plants and invertebrates, and to possess various biological activities such as protein kinase C-dependent signal transduction, regulation of gene expression and cell proliferation (see review of Aitken *et al.*, 1992). As for the tissue distribution of 14-3-3 isoforms, isoforms are expressed in most tissues, but are abundant in brain tissue (Watanabe *et al.*, 1993). Recently, the partial cDNA of 14-3-3β was isolated from the pituitary gland of the frog, *Xenopus laevis* (Martens *et al.*, 1992), but it completely lacked the 3'-untranslated region (UTR). Our original interest was that this protein activates protein kinase C on signal transduction. Thus, a frog cDNA homologue of 14-3-3 protein was isolated and its primary structure was determined. Here we report that the cDNA homologue isolated is the ζ type of 14-3-3 protein, and that the two highly homologous sequences exist in the 3'-UTR of frog and human 14-3-3ζ cDNA.

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MATERIALS AND METHODS

Animals

The frog, *Rana rugosa* was used for all experiments. At 20 hr before obtaining unfertilized eggs, frogs were primed by injecting the extract of pituitaries of *Rana catesbeiana* into the body cavity by the method of Kashiwagi and Kashiwagi (1993). Tadpoles were staged according to Shumway (1940), and Taylor and Kollros (1946).

Cloning of the frog ζ 14-3-3 cDNA

To isolate cDNA encoding the 14-3-3ζ, RT-PCR was employed with the forward and backward primers corresponding to nucleotides 344-364 (F1) and 814-838 (B1) of rat 14-3-3ζ cDNA, respectively [(F1), 5'-CTGAGAAAAAGCAGCAGATGGC-3'; (B1) 5'-ATCTCATAG-TAGAACACAGAGAA-3']. The PCR products obtained were cloned into the M13mp18 cloning vector. The nucleotide sequence of the PCR product had 82% identity with that of rat 14-3-3ζ cDNA (Watanabe *et al.*, 1993). Thus, this PCR fragment was used as a probe to isolate cDNA encoding frog 14-3-3ζ. A λgt10 cDNA library was then constructed from poly(A)$^+$ RNA of whole tadpoles at stage 25 as described previously (Takase *et al.*, 1992). Whole tadpole λgt10 library was screened with the DIG-labeled PCR product (0.4 kbp) as a probe. Hybridization was performed according to the manufacturer's protocol (Boehringer Mannheim). Inserts from λ phages hybridized to the probe were subcloned into the phage vector M13mp18. The cDNA clones were sequenced on both DNA strands using the ABI 373A

automated DNA sequencer following the manufacturer's guide (Perkin Elmer).

Northern blot analysis

Whole tadpoles at stage 25 and various tissues of adult frogs were used to isolate total RNA. RNA (15 μg/lane) was electrophoresed on a 1.2% denaturing formaldehyde agarose gel and transferred to nylon membranes (Hybond N; Amersham). RNA was then hybridized with the DIG-labelled 0.7- kbp *Eco*RI/*Hinc* II fragment in the 3'UTR of the frog 14-3-3ζ cDNA. The DIG DNA labeling kit and DIG luminescent detection kit (Boehringer Mannheim) were used for this analysis following the manufacturer's directions.

Southern blot analysis of RT-PCR products

All RNA prepared from different tissues of adult frogs were used as the initial templates for RT-PCR. A 431 bp-fragment of the 3'-UTR of the 14-3-3ζ cDNA was amplified by the PCR using the forward and backward primers, 5'-CCATTGTCATCCTTACTGTCC-3' corresponding to base pairs 889-909 of the 14-3-3ζ cDNA and 5'-TGGCTATCACAGGAATCACTCA-3' corresponding to base pairs 1299-1321 of the 14-3-3ζ cDNA. The PCR products (0.4 kbp) were electrophoresed on 0.8% agarose gel and transferred to nylon membranes (GeneScreen™; NEN Research Products). The DNA was then hybridized with DIG-labeled 14-3-3ζ cDNA (1.8 kbp) as mentioned earlier.

RESULTS

Primary structure

Screening of the frog cDNA library produced 4 positive clones. All clones had the open reading frame of the 14-3-3ζ cDNA. As shown in Fig. 1, the cDNA had 1,774 nucleotides. A total of 1,774 nucleotides was present with 103 nucleotides in the 5'-UTR, 245 residues from which the molecular weight was calculated to be 28,028, and 936 bases in the 3'-UTR. The nucleotide sequence of frog 14-3-3ζ cDNA was 75% identical with rat 14-3-3ζ cDNA, 67% human PLA2, which is now known as 14-3-3ζ (Zupan *et al.*, 1992; Du *et al.*, 1994), and 74% with *Xenopus* 14-3-3, but 55%, 53%, 54%, and 55% with

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                                                        GGACCGGACTCTCCTCCCAGCGGCA   25
GCAGCCAGAGAGACGCGCATTCCGCCCAGCCCCGGGGACCCCGACCGCCAGCAGCTCTCCGCCACAGAACCATTAGCC  103
ATGGATAAAAACGAGCTGGTCCAGAAAGCCAAATTGGCCGAGCAGGCAGAGCGGTACGATGACATGGCAGGCTGCATG  181
M  D  K  N  E  L  V  Q  K  A  K  L  A  E  Q  A  E  R  Y  D  D  M  A  G  C  M    26

AAAAAAGTGACGGAACAAGGAACTGAACTATCCAATGAGGAGAGGAACCTTCTCTCGGTGGCCTACAAAAATGTAGTA  259
K  K  V  T  E  Q  G  T  E  L  S  N  E  E  R  N  L  L  S  V  A  Y  K  N  V  V    52

GGTGCACGCCGGTCATCCTGGAGAGTTGTCTCTAGTATTGAGCAAAAAACAGAAGGAAGTGATAAAAAACAGCAGATG  337
G  A  R  R  S  S  W  R  V  V  S  S  I  E  Q  K  T  E  G  S  D  K  K  Q  Q  M    78

GCTCGGGAGTATCGGGAGAAGATTGAAAAGGAGCTGAGAGATATTTGTACAGATGTGCTGCTTCTCCTGGAAAAATTC  415
A  R  E  Y  R  E  K  I  E  K  E  L  R  D  I  C  T  D  V  L  L  L  L  E  K  F   104

TTGATCCCCAATGCATCCCAAGCAGAGAGCAAAGTCTTCTACTTGAAAATGAAGGGAGACTACTACCGTTACTTGGCT  493
L  I  P  N  A  S  Q  A  E  S  K  V  F  Y  L  K  M  K  G  D  Y  Y  R  Y  L  A   130

GAAGTAGCTAGCGGAGACAATAAAAAAACAATTGTGGATCAGTCTCAGGAGGCATACCAGGAGGCTTTTGATATCAGC  571
E  V  A  S  G  D  N  K  K  T  I  V  D  Q  S  Q  E  A  Y  Q  E  A  F  D  I  S   156

AAACGGGAGATGCAACCAACACATCCCATAAGGCTGGGCCTGGCTCTGAACTTCTCTGTCTTCTATTACGAGATCCTG  649
K  R  E  M  Q  P  T  H  P  I  R  L  G  L  A  L  N  F  S  V  F  Y  Y  E  I  L   182

AACTCCCCGGAAAAGGCGTGCGCTCTGGCAAAAACTGCCTTTGACGAAGCTATTGCCGAACTCGATACCCTAAGCGAA  727
N  S  P  E  K  A  C  A  L  A  K  T  A  F  D  E  A  I  A  E  L  D  T  L  S  E   208

GAATCATACAAAGACAGCACATTAATAATGCAATTACTGAGAGACAATTTGACATTGTGGACATCAGACACTCAAGGA  805
E  S  Y  K  D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W  T  S  D  T  Q  G   234

GATGAAGCCGAAGCAGAAGTCCCCACAGAAAATTAATTGGCCTTCCTTTTCTGTCTGCCTCATTCTAAAATTTCCACA  883
D  E  A  E  A  E  V  P  T  E  N  *                                              245

ATAGACCATTGTCATCCTTACTGTCCCACACATAGGTTTATTTTTTTTATTTTATTTTTTTTGTTTACAATTATGAAA  961
GGTTTATGTTACTTCTATTTGGATTTCTATATTTCCCATGTGGTTTTGTGTTTAATAGGGAGTAGGGCCAGTTAACAT 1039
TTTGGGGGGTCAATTTTTTTTCCAACCTCATAATGTGACCAATATGGGGAATATGGAATTTTTATACAGGTTTTAAAT 1117
GTTTGGCACAGTACTTCTGGTACATTGTAGGTTACCACAGGGCCCGATGTTTAATCAGATTCCCATATTTAAAAGGTG 1195
ACCAACTTGTGTATTACTGTAACTCCAAAAAAAAGGGGGCATTTGGTTAGCGCTGTAGGTAGAGACAATAAACTCCTA 1273
CCTTTAGCACAGCCATACATATTCATTGAGTGATTCCTGTGATAGCCAATCAAAATCACGAGTGCAAAACGGTTCACA 1351
TTCCATTCTCACAGTTTAGCTAAGGTTTGCTTTTGTTTGTTGCTATTCTCCATTTTATTTGTATTTAAATTGTTTAAA 1429
ACGACCTAAGATCAAAAAAAAGAGAAGTACTTGATACATTTAAAGATGCATACATGCTTGGTATCCATTAAGTCACTA 1507
TATATATCAAGCACAGCAGAAAAAAGAGAAAACCCATATATTTAACTTTTAGTTTTATTTTTTGTTTTTGATACTTGC 1585
CTAACATGCATGTGGCTGTAAAAATACAATACAATATAGTTAGCAGGGGAAATAACTTGAGATGATGGCTAGCTTTGT 1663
TTAATGTCTTATGAAATTTTCATGAACAATCCAAGCATAATTGTTAAAGAACATGTGTAATAAGTTGATGTAAGTGGA 1741
ATAAAAGTTTTATGAATGGACTTTTCAACTACT                                              1774
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Fig. 1. Nucleotide and deduced amino acid sequences of the 14-3-3ζ gene from the frog, *R. rugosa*. The nucleotide and amino acid sequences are numbered from the first nucleotide and the initiator methionine codon on the left of each line, respectively. Asterisk indicates the stop codon. The polyadenylation signal (AATAAA) is single-underlined.

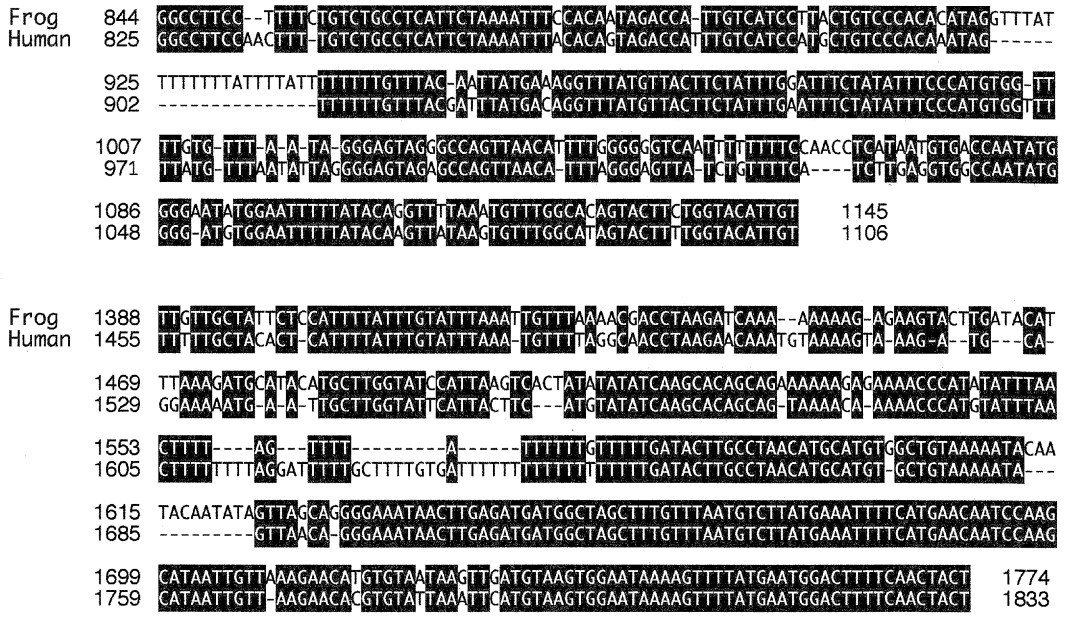

Fig. 2. Comparisons of 3'-untranslated sequences for frog and human 14-3-3ζ cDNA. Alignment of 3'-untranslated cDNA sequences for frog and human 14-3-3ζ mRNA was made. Nucleotide sequences of frog (this work) and human 14-3-3ζ (Zupan *et al.*, 1992) are numbered from the first nucleotide on the left of each line as shown in Fig. 1. To maximize homologies, gaps represented by hyphenes are introduced in the two sequences.

rat 14-3-3β, γ, η and θ, respectively. However, a striking homology (92%) was found at the amino acid level among frog, rat, bovine and human 14-3-3ζs. A comparison of the amino acid sequence of frog 14-3-3ζ with those of rat subtypes of 14-3-3 (β, γ, ε, η and θ) showed that there was a lower homology (83%, 72%, 67%, 72% and 79% sequence identity for the β/ζ, γ/ζ, ε/ζ, η/ζ and θ/ζ subtype-pairs, respectively), but no significant homology in the 3'-UTR of these subtype pairs. However, a high homology was found in the 3'-UTR of 14-3-3ζ cDNA of frogs and humans (Fig. 2). Alignment of the nucleotide sequenses in the 3'-UTR of frog and human 14-3-3ζ cDNA showed two highly conserved regions at nucleotide positions 844-1145 (79% identity) and 1388-1774 (79% identity), although gaps were introduced between two sequences to maximize homologies (Fig. 2).

Northern blot analysis

Northern blot analysis showed that 14-3-3ζ mRNA was expressed in tadpoles at stage 25 with a signal of about 1.8 kb (Fig. 3).

Expression of 14-3-3ζ in various tissues of adult frogs

RT-PCR was employed to examine the 14-3-3ζ gene expression in various tissues of adult frogs. After agarose gel electrophoresis, the transcripts (0.4 kbp) amplified by RT-PCR were detected by Southern blot analysis. As can be seen in Fig. 4, the 14-3-3ζ mRNA level was high in the brain, lung, spleen and kidney, but low in the heart and testis. Little mRNA was detected in the liver, pancreas, ovary or muscle.

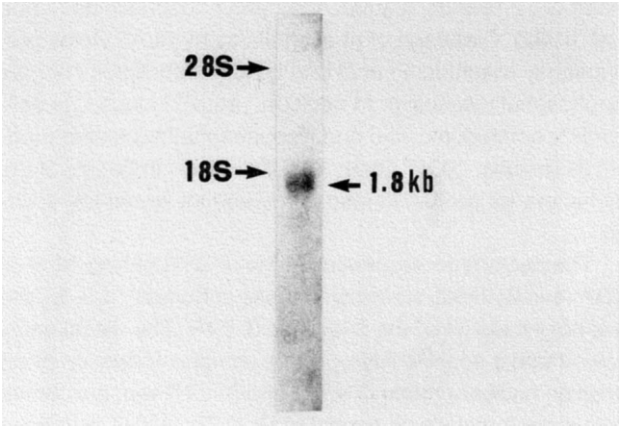

Fig. 3. Expression of the 14-3-3ζ mRNA in tadpoles. Total RNA was isolated from whole tadpoles at stage 25. The number on the right side of panel indicates the size of a transcript. The position of 28S and 18S ribosomal subunits is indicated with arrows.

DISCUSSION

In this study we cloned and sequenced frog 14-3-3ζ cDNA. At least 7 isoforms of the 14-3-3 protein are known, including representatives of mammalian, amphibian, insect, plant and yeast origin. Frog and human 14-3-3ζ were 67% identical at the nucleotide level, but the open reading frame of frog and human 14-3-3ζ were 82% identical. Comparing the two amino acid sequences indicated that frog 14-3-3ζ isoform differs at only 19 out of 245 residues (92% identity) from the human 14-

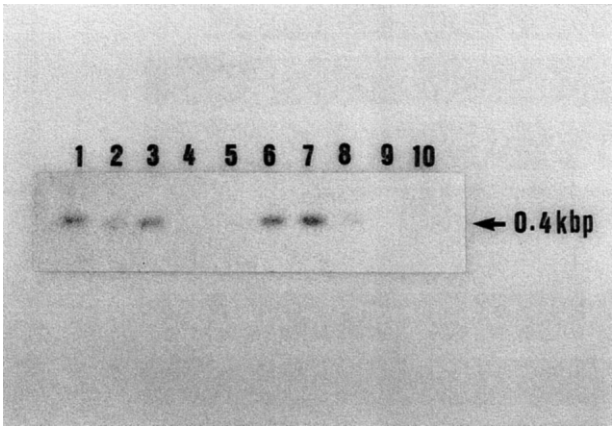

Fig. 4. Southern blot analysis of RT-PCR products. Southern blot analysis was carried out as described in MATERIALS AND METHODS. Each lane represents the brain (1), heart (2), lung (3), liver (4), pancreas (5), spleen (6), kidney (7), testis (8), ovary (9), and muscle (10).

3-3ζ sequence. These results suggest that this protein may play the same role in cellular physiology in both amphibians and mammals. 14-3-3 proteins function in several different ways as described earlier. Interestingly, human 14-3-3ζ has PLA2 activity (Du *et al.*, 1994). *In vitro* activation of protein kinase C by 14-3-3ζ requires Ca^{2+} and phospholipids (Isobe *et al.*, 1992). Cleavage of phospholipids by PLA2 yields predominantly arachidonic acid and lysophospholipids such as lysophosphatidylcholine. In addition, protein kinase C is activated by arachidonic acid and lysophosphatidylcholine products (Nishizuka, 1992). Thus, 14-3-3ζ is likely to be one of the key factors for protein kinase C-dependent signal transduction.

The nucleotide sequences in the 3'-UTR of frog 14-3-3ζ cDNA revealed high similarity to those of human 14-3-3ζ. We have not yet identified the role of the 3'-UTR. The recent study showed that a specific RNA-protein complex formation of rat transition nuclear protein (TNP) 2 and 3'-UTR was the central event in the translational control of rat TNP2 mRNA (Schlicker *et al.*, 1997). The 8 nucleotide-motif in the 3'-UTR of mouse protamine 1 was necessary for temporal and spacial expression of the gene (Braun *et al.*, 1989; Braun, 1990). An 18 kDa protein acted as a repressor of mouse basic chromosomal protein mRNA translation by interacting with highly conserved elements in its 3'-UTR (Kwon and Hecht, 1993). In view of these findings, it is speculated that the homologous sequence in the 3'-UTR may be required for RNA-binding proteins, and is essential for the translational control of both frog and human 14-3-3ζ mRNAs. Amino acid sequences of a RNA-binding protein(s), if they exist, are presumably highly homologous between frog and human.

When the amino acid sequence of frog 14-3-3ζ was aligned with those of other known 14-3-3 proteins by the UPGMA method (Felsenstein, 1993), extensive sequence similarities were seen (Fig. 5). 14-3-3γ and η are closer to the

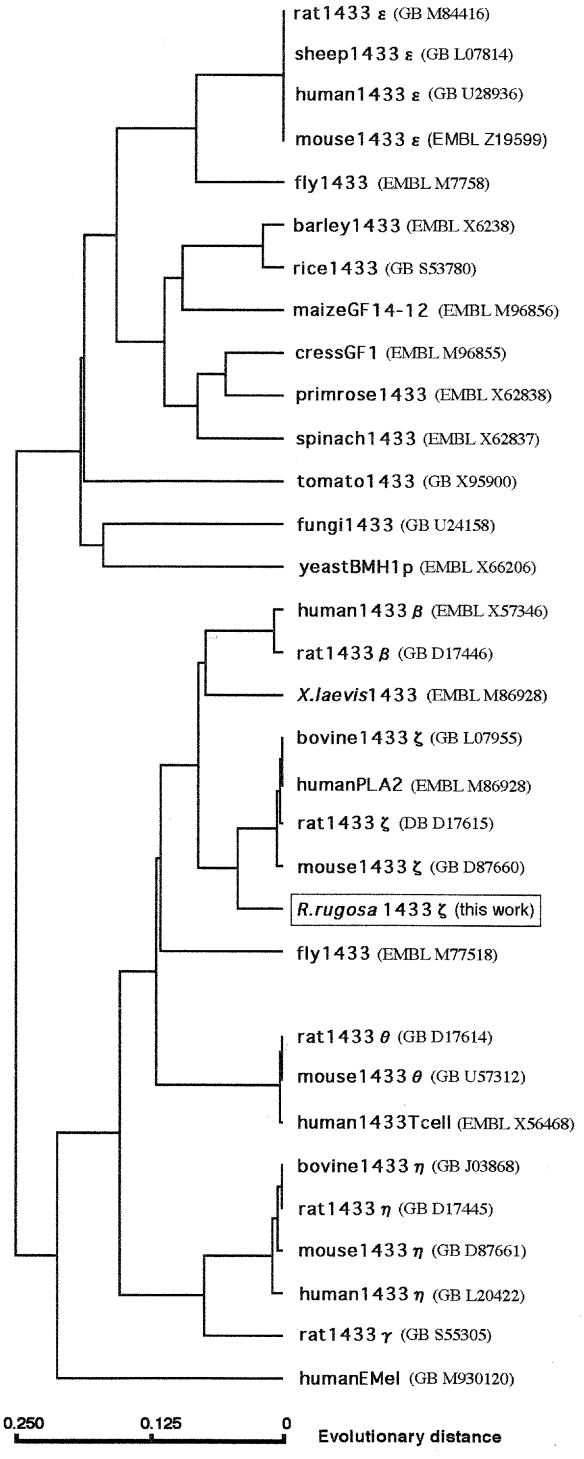

Fig. 5. A phylogenetic tree of frog 14-3-3ζ and other known 14-3-3 proteins. The data base source and the accession number are given in parenthesis. The phylogenetic tree was constructed by the UPGMA method (Felsenstein, 1993). GB, GenBank™.

β and ζ sequences. Yeast and plant sequences are closer to the ε subtype. *R. rugosa* 14-3-3ζ was 79% identical with *X. laevis* 14-3-3 (Martens *et al.*, 1992) , and 92% with rat 14-3-3ζ, indicating that the *R. rugosa* sequence is closer to rat rather

than *X. laevis*. This is reasonable because *X. laevis* 14-3-3 protein has the β subtype. Judged from the phylogenetic tree, it can be concluded that the 14-3-3 protein family evolved and diverged before the evolutionary separation of plants and animals into different species.

The ζ subtype mRNA was distributed widely in various regions of rat brain tissue (Watanabe *et al.*, 1994). From these and other findings, Watanabe *et al.* (1994) hypothesized that 14-3-3ζ might be associated with different functional regulations and involvements in neurons. In this study, the 14-3-3ζ mRNA level was found to be high in the brain, lung, spleen and kidney, but low in the heart and testis. According to their report (Watanabe *et al.*, 1993), β, γ and η subtypes of 14-3-3 protein were expressed abundantly in the brain at the transcription level. The expression of these three subtypes was low in the ovary, and little expression was seen in the testis. These results are incompatible with those of Watanabe *et al.* (1993), because our 14-3-3 protein is the ζ type while their's had other types. Expression levels in frog tissues may be variable depending on the subtypes of the 14-3-3 protein. Frog 14-3-3ζ may play a role(s) similar to others in different species. Furthermore, homologous sequences in the 3'-UTR of frog and human 14-3-3ζ genes might be required for the translational control of their mRNA. Further investigation will, of course, be required to prove this hypothesis.

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