

cDNA cloning reveals a tissue specific expression of alternatively spliced transcripts of the ryanodine receptor type 3 (RyR3) calcium release channel

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Abstract Ryanodine receptors (RyRs) are a family of intracellular calcium release channels. Three cDNAs encoding different isoforms of RyR have been identified and cloned. We report the complete sequence of the mink RyR3 cDNA and the characterization of three alternative spliced regions. The first two splicing sites are represented by insertions of five and six amino acids, respectively. The third site is represented by a mutually exclusive splicing. The tissue distribution of the alternatively spliced transcripts revealed a ubiquitous expression of splicing site I and a differential distribution of sites II and III, indicating that a further level of complexity in RyR3 expression may result from alternative splicings in this gene.

Key words: Ryanodine receptor; Alternative splicing

1. Introduction

Two classes of intracellular channels that play a key role in regulating calcium release from intracellular stores have so far been identified: the inositol 1,4,5-trisphosphate (InsP₃) receptors and the ryanodine receptors (RyR) [1–4]. Ryanodine receptors are mainly known for their role in regulating calcium release from the sarcoplasmic reticulum of cardiac and skeletal muscle [4–6]. A third isoform, RyR3, has been proved to be widely expressed in different tissues and may, therefore, play an important role in calcium homeostasis, also in non-excitable cells [7–10]. Further studies have pointed out that also RyR1 and RyR2 are expressed in tissues other than skeletal and cardiac muscles. Interestingly, more than one isoform can be detected in the same cell type, indicating that ryanodine receptors have a relatively large heterogeneity in their expression pattern [8].

Similar to the InsP₃ type 1 receptor [11,12], new findings suggest that further heterogeneity in the RyR family may result from alternative splicings of mRNA. Two alternative splicing sites have been localized in human and mouse RyR1 mRNA. Insertions of 5 amino acids (Ala-Gly-Asp-Ile-Gln) after residue 3489 and of 6 amino acids (Val-Ile-Asn-Arg-Gln-Asn) after residue 3864 have been detected in the

human RyR1 cDNA by Zhang et al. [13]. In the mouse RyR1 cDNA, Futatsugi et al. found a similar 5 amino acid insertion at the same position reported for the human RyR1, with a conservative substitution of Ile at position 4 with Val, and the same 6 amino acid insertion at residue 3865 [14]. A putative alternative splicing site, comprising residues 11146–11169, has been postulated in the rabbit RyR2 mRNA [6] and a second one has been described by Anthony Lai and co-workers [15]. While sequencing the full length of the mink RyR3 cDNA, we have identified three alternative splicing sites. Two of them are similar to those identified by Futatsugi et al. and by Zhang et al. in RyR1, while the third is a novel site. Interestingly, also this third site, like the other two, is localized in the putative modulatory region. The tissue distribution of the three splicing subtypes has been analyzed.

2. Materials and methods

2.1. DNA and RNA extraction and RT-PCR

Genomic DNA from CCL64 cells was prepared as described by Sambrook et al. [16]. Total RNA for cDNA library synthesis was prepared as described [7,17]. 10 µg of total RNA was retrotranscribed using a RyR3 specific primer 2.5 µM (5'-CTTCCAGGAGGGACAGAAG-3', nucleotides 11596–11614), in a buffer containing 50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM dithioerythritol, pH 8.5, 1 µM each of dCTP, dGTP, dTTP, and dATP, 200 U of M-MuLV reverse transcriptase, in a volume of 20 µl. The reaction was performed at 37°C for 1 h. 2 µl of the reverse transcription reaction mix was used for amplification in a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9, 0.1% Triton X-100, 1.5 mM MgCl₂, 200 µM of each dNTP, 1 mM oligonucleotide PCR primers and 2.5 U of Taq DNA polymerase, in a final volume of 50 µl. The samples were subjected to 20 cycles of amplification consisting of a denaturation step of 30 s at 94°C, an annealing step of 30 s at 54°C or 63°C, and an elongation step of 45 s at 72°C, followed by a final extension step at 72°C for 5 min. The PCR reaction mixture was diluted 1:100 and subjected to 20 cycles of amplification using a nested primer. The following couples of primers were used for the amplification of the three splicing sites of RyR3.

Alternative splicing site I: 5'-AGTCCCAGTGCTGACTCTGA-3' (PR101, nucleotides 9841–9860) and 5'-TCCCTA-TAGCTGTATCGAGA-3' (PR102RC, nucleotides 10162–10181);

Alternative splicing site II: 5'-TGAGCCCCATGGTGGTTGAG-3' (PR103, nucleotides 10904–10923) and 5'-AGGACCGAAAGAGATCAGC-3' (PR104RC, nucleotides 11161–11180);

Alternative splicing site III: 5'-CCTCTCTACAACCTGCC-CAGACA-3' (PR93, nucleotides 10462–10485) and 5'-GGTA-TAAGCCTGTCATACTGAGT-3' (PR98RC, nucleotides 10549–10571) designed to amplify exon A or 5'-CAAAGGAA-

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Abbreviations: RyR, ryanodine receptor; AS, alternative splicing; InsP₃, inositol 1,4,5-trisphosphate; CaM, calmodulin

TACTCTCTGTT-TCTA-3' (PR114RC) designed to amplify exon B.

Amplification products relative to the alternative splicing sites I and II were digested with *MseI* or *NaeI* restriction enzymes, respectively, and analyzed on 3% NuSieve, 0.5% Agarose gel.

2.2. Cloning and sequencing of RT-PCR products

Amplified DNA fragments corresponding to the alternative splicing sites I and II were cloned into *EcoRI/BamHI* sites of the pBluescript vector. The alternative splicing site III PCR products were cloned into *SmaI/EcoRV* sites of pBluescript. Both strands were then sequenced with the Sequenase Version 2.0 kit (Amersham) according to the manufacturer's instructions.

3. Results

3.1. Cloning and cDNA sequence determination

To clone the full-length mink RyR3 cDNA we used a size selected oligo-dT cDNA library obtained from RNA extracted from mink lung epithelial cells treated with TGF- β , as described [7]. This library was screened with the 5' sequence of the original RyR3 clone β 4 we previously isolated [7]. The first round of screening resulted in the isolation of a clone of 5.3 kb (GM1), spanning nucleotides 9774–15068. The 5' end sequence of GM1 was used to screen a random primed cDNA library (from the same mRNA as above), resulting in the isolation of a new clone, GM28 (8758–9865). The same strategy led to the isolation of other two clones spanning the regions corresponding to nucleotides 6307–8504 and 7817–9131, respectively.

In order to obtain the remaining 5' of the mink RyR3 cDNA, the RT-PCR technology was used. Single strand cDNA was synthesized from 10 μ g of poly(A)⁺ RNA, primed with a complementary oligonucleotide designed from the 5' sequence of the upstream clone obtained. PCR amplification was carried out using as reverse primer the same oligonucleotide used for the RT reaction and as forward primer degenerated oligonucleotides that were designed considering the more conserved regions between rabbit RyR1, RyR2 and RyR3 amino acid sequences and selecting for amino acids with minimal degeneracy in their codons. This strategy led to the isolation of eight clones which cover the remaining part of the RyR3 nucleotide sequence, up to the initiating ATG codon (Fig. 1).

The complete nucleotide and deduced amino acid sequences of the mink RyR3 cDNA were determined by sequence analysis of the 12 described overlapping clones. The entire cDNA isolated is 15068 base pairs in length, the termination codon (TAA) was found 14578 bases after the initiating ATG. The 3' untranslated region of the cDNA is 491 nucleotides long (excluding the poly dA tract) with a canonical AAATAAA polyadenylation signal only 47 bases downstream of the ter-

mination codon. The amino acid sequence deduced from the nucleotide sequence is 4859 amino acids long, with a calculated molecular weight of 550769 (including the first methionine). The amino acid sequence of the mink RyR3 was compared to that of rabbit RyR1 and RyR2 revealing an overall amino acid sequence identity of 69.4% and 69.5%, respectively.

When compared with chicken, frog and rabbit RyR3 sequences, the mink RyR3 protein revealed an identity of 86.94, 86.22 and 92.6%, respectively. The amino acid sequence of the mink RyR3 and the comparison with other known RyR3 sequences are reported in Fig. 2.

3.2. Analysis of potential regulatory and modification sites

Analogous to other RyR sequences, the mink RyR3 also contains 4 internal repeats (849–934, 965–1049, 2596–2681, 2713–2793). The C-terminal of the mink RyR3 contains 12 stretches rich in hydrophobic amino acids, which potentially form transmembrane (TM) domains, as also observed in the sequence of other RyRs (2983–3003, 3047–3065, 3834–3854, 3862–3880, 4119–4142, 4184–4204, 4399–4420, 4471–4494, 4611–4642, 4659–4678, 4701–4120, 4736–4759). Out of the 12 domains (named M', M'' and M1–M10), two models based on structure prediction algorithms have been proposed, the first containing 4 transmembrane domains (4399–4420, 4471–4494, 4659–4678, 4736–4759) that have a high probability to pass through the membrane, while the second, in addition to the previous 4, includes 6 more regions (3834–3854, 3862–3880, 4119–4142, 4184–4204, 4611–4642, 4701–4120) for a total of 10 (M1–M10).

Several potential binding sites for regulatory compounds can be identified in the primary sequence of RyR3. Computer block search on mink RyR3 protein sequence has revealed the presence of a sequence resembling an E-F hand motif located between residues 3914 and 3932 [18]. A consensus sequence GXGXXG for adenine nucleotide binding was predicted to lie in residues 697–702, 699–704, 1134–1139, 2234–2239, 2521–2526. With the exception of the one encompassing the region between residues 1134–1139 that is common only to the chicken RyR3, the other three are conserved in all the RyR3 known sequences. Using the consensus sequence RXXS/T for the potential phosphorylation sites of the Ca²⁺ CaM-dependent protein kinase, 21 putative sites were found. Three of these sites (127–130, 287–290, 2703–2706) are present in all known RyR1, RyR2 and RyR3 proteins, while 7 other sites are conserved only in the RyR3 isoform. Interestingly, a potential PKA phosphorylation site (T-1243), absent in RyR1 and RyR2, is conserved in mink, rabbit, chicken and frog RyR3. One potential phosphorylation site for cyclic AMP-dependent protein kinase KRXXS/T is found at positions 1239–1243 and is conserved in all RyR3 amino acid sequences.

A potential glycosylation site at residues 4686–4688 conserved in all RyRs may be exposed in the lumen of the endoplasmic reticulum.

3.3. Identification of three alternative splicing sites in the mink RyR3 mRNA

Two alternative splicing sites in the mouse and human RyR1 mRNA have been originally described by Zhang et al. and Futatsugi et al. [13,14]. In order to verify the presence of the corresponding splicing sites in RyR3 mRNA, we de-

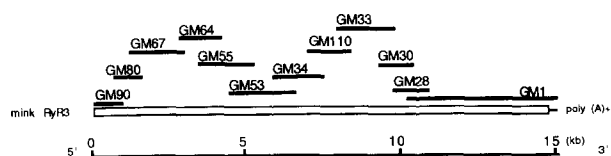


Fig. 1. Structure of the mink RyR3 mRNA. The coding region of the RyR3 mRNA is represented by an open box and the 3' untranslated region by a solid line. The original clones isolated and sequenced are reproduced at the top of the figure.

mkRyR3	1	MAEGGEGGEDE	1	IFLRTDEEVVLQCI	AN	HKQKRFKCLAAEGLGNRLCFLEPTSEAKYVPPDL	CN	FNLEQSLSVRALQEMLANTG	ENGEGGAAGGGGH	100
mkRyR3	1	MAEGGEGGEDE	1	IFLRTDEEVVLQCI	AN	HKQKRFKCLAAEGLGNRLCFLEPTSEAKYVPPDL	CN	FNLEQSLSVRALQEMLANTG	ENGEGGAAGGGGH	100
mkRyR3	1	MAEGGEGGEDE	1	IFLRTDEEVVLQCI	AN	HKQKRFKCLAAEGLGNRLCFLEPTSEAKYVPPDL	CN	FNLEQSLSVRALQEMLANTG	ENGEGGAAGGGGH	100
mkRyR3	1	MAEGGEGGEDE	1	IFLRTDEEVVLQCI	AN	HKQKRFKCLAAEGLGNRLCFLEPTSEAKYVPPDL	CN	FNLEQSLSVRALQEMLANTG	ENGEGGAAGGGGH	100
mkRyR3	101	RTLLYGHAILLRHSFSGMYLTC	101	LTSSRSOTDKLAFDVG	REHATGEACWWT	HPASKQRSEGEKVRIGDGL	ILVSVSSERYLHLSVSN	...	IGNVOVDS	198
mkRyR3	101	RTLLYGHAILLRHSFSGMYLTC	101	LTSSRSOTDKLAFDVG	REHATGEACWWT	HPASKQRSEGEKVRIGDGL	ILVSVSSERYLHLSVSN	...	IGNVOVDS	198
mkRyR3	101	RTLLYGHAILLRHSFSGMYLTC	101	LTSSRSOTDKLAFDVG	REHATGEACWWT	HPASKQRSEGEKVRIGDGL	ILVSVSSERYLHLSVSN	...	IGNVOVDS	198
mkRyR3	101	RTLLYGHAILLRHSFSGMYLTC	101	LTSSRSOTDKLAFDVG	REHATGEACWWT	HPASKQRSEGEKVRIGDGL	ILVSVSSERYLHLSVSN	...	IGNVOVDS	198
mkRyR3	199	QTLWNVHPTCSGSS	199	IEEGYLLGGHVRLFHGHDECLT	IPSTQDNDQARR	FYEAGGAGTRASLRWRVPL	RI	SWSGSNI	IRWGQDFLRHIT	298
mkRyR3	199	QTLWNVHPTCSGSS	199	IEEGYLLGGHVRLFHGHDECLT	IPSTQDNDQARR	FYEAGGAGTRASLRWRVPL	RI	SWSGSNI	IRWGQDFLRHIT	298
mkRyR3	199	QTLWNVHPTCSGSS	199	IEEGYLLGGHVRLFHGHDECLT	IPSTQDNDQARR	FYEAGGAGTRASLRWRVPL	RI	SWSGSNI	IRWGQDFLRHIT	298
mkRyR3	199	QTLWNVHPTCSGSS	199	IEEGYLLGGHVRLFHGHDECLT	IPSTQDNDQARR	FYEAGGAGTRASLRWRVPL	RI	SWSGSNI	IRWGQDFLRHIT	298
mkRyR3	299	EDQGLLLQDRAKSOTKSTAFS	299	FKASKETKEKLDSSHKRD	IEQMGVPEIKYGD	SVCFVQHI	ASGLWVTKA	QDAKTS	SLGPK	398
mkRyR3	299	EDQGLLLQDRAKSOTKSTAFS	299	FKASKETKEKLDSSHKRD	IEQMGVPEIKYGD	SVCFVQHI	ASGLWVTKA	QDAKTS	SLGPK	398
mkRyR3	299	EDQGLLLQDRAKSOTKSTAFS	299	FKASKETKEKLDSSHKRD	IEQMGVPEIKYGD	SVCFVQHI	ASGLWVTKA	QDAKTS	SLGPK	398
mkRyR3	299	EDQGLLLQDRAKSOTKSTAFS	299	FKASKETKEKLDSSHKRD	IEQMGVPEIKYGD	SVCFVQHI	ASGLWVTKA	QDAKTS	SLGPK	398
mkRyR3	399	RCQREESQAARI	399	IRNTTALFSQFVS	...	GNRTA	APVLP	PI	IEVQ	491
mkRyR3	399	RCQREESQAARI	399	IRNTTALFSQFVS	...	GNRTA	APVLP	PI	IEVQ	491
mkRyR3	399	RCQREESQAARI	399	IRNTTALFSQFVS	...	GNRTA	APVLP	PI	IEVQ	491
mkRyR3	399	RCQREESQAARI	399	IRNTTALFSQFVS	...	GNRTA	APVLP	PI	IEVQ	491
mkRyR3	491	LNINYSVAHFAGI	491	AREESQMAWKE	ILNLYKLLAAL	IRGNRNCAQSN	NL	DL	ISKDL	591
mkRyR3	491	LNINYSVAHFAGI	491	AREESQMAWKE	ILNLYKLLAAL	IRGNRNCAQSN	NL	DL	ISKDL	591
mkRyR3	491	LNINYSVAHFAGI	491	AREESQMAWKE	ILNLYKLLAAL	IRGNRNCAQSN	NL	DL	ISKDL	591
mkRyR3	491	LNINYSVAHFAGI	491	AREESQMAWKE	ILNLYKLLAAL	IRGNRNCAQSN	NL	DL	ISKDL	591
mkRyR3	591	GRNHKVLQDLVCLSC	591	LCNGVAVRANONLI	CDNLLPRNLLQTL	IRNDVTS	IRPN	IFL	GA	691
mkRyR3	591	GRNHKVLQDLVCLSC	591	LCNGVAVRANONLI	CDNLLPRNLLQTL	IRNDVTS	IRPN	IFL	GA	691
mkRyR3	591	GRNHKVLQDLVCLSC	591	LCNGVAVRANONLI	CDNLLPRNLLQTL	IRNDVTS	IRPN	IFL	GA	691
mkRyR3	591	GRNHKVLQDLVCLSC	591	LCNGVAVRANONLI	CDNLLPRNLLQTL	IRNDVTS	IRPN	IFL	GA	691
mkRyR3	691	PYPGGGEGWAGNGV	691	GDYLSYGF	DGLHLWSGRI	IPRAVAS	IN	CHL	ISKDD	791
mkRyR3	691	PYPGGGEGWAGNGV	691	GDYLSYGF	DGLHLWSGRI	IPRAVAS	IN	CHL	ISKDD	791
mkRyR3	691	PYPGGGEGWAGNGV	691	GDYLSYGF	DGLHLWSGRI	IPRAVAS	IN	CHL	ISKDD	791
mkRyR3	691	PYPGGGEGWAGNGV	691	GDYLSYGF	DGLHLWSGRI	IPRAVAS	IN	CHL	ISKDD	791
mkRyR3	791	GGHGEKFLPPSGYAP	791	CEYALLPKEKMLPE	VPKYEKRD	AEIGRDLLGTT	CS	AS	FI	891
mkRyR3	791	GGHGEKFLPPSGYAP	791	CEYALLPKEKMLPE	VPKYEKRD	AEIGRDLLGTT	CS	AS	FI	891
mkRyR3	791	GGHGEKFLPPSGYAP	791	CEYALLPKEKMLPE	VPKYEKRD	AEIGRDLLGTT	CS	AS	FI	891
mkRyR3	791	GGHGEKFLPPSGYAP	791	CEYALLPKEKMLPE	VPKYEKRD	AEIGRDLLGTT	CS	AS	FI	891
mkRyR3	891	GKIRDDNNKQHGL	891	VEFSKLPET	EKNYLMQSTETL	KTLALGCH	I	AHNP	AAEED	991
mkRyR3	891	GKIRDDNNKQHGL	891	VEFSKLPET	EKNYLMQSTETL	KTLALGCH	I	AHNP	AAEED	991
mkRyR3	891	GKIRDDNNKQHGL	891	VEFSKLPET	EKNYLMQSTETL	KTLALGCH	I	AHNP	AAEED	991
mkRyR3	891	GKIRDDNNKQHGL	891	VEFSKLPET	EKNYLMQSTETL	KTLALGCH	I	AHNP	AAEED	991
mkRyR3	991	NVWAKDRIKQGWY	991	GIQDLKKNRNP	LPVYAL	LDERTKSNRDS	LA	EA	VRT	1091
mkRyR3	991	NVWAKDRIKQGWY	991	GIQDLKKNRNP	LPVYAL	LDERTKSNRDS	LA	EA	VRT	1091
mkRyR3	991	NVWAKDRIKQGWY	991	GIQDLKKNRNP	LPVYAL	LDERTKSNRDS	LA	EA	VRT	1091
mkRyR3	991	NVWAKDRIKQGWY	991	GIQDLKKNRNP	LPVYAL	LDERTKSNRDS	LA	EA	VRT	1091
mkRyR3	1091	EVVTTGGDMRVG	1091	WARPGCRPD	IELGADDA	AFVFGSGR	GHWRGSGY	FP	GW	1191
mkRyR3	1091	EVVTTGGDMRVG	1091	WARPGCRPD	IELGADDA	AFVFGSGR	GHWRGSGY	FP	GW	1191
mkRyR3	1091	EVVTTGGDMRVG	1091	WARPGCRPD	IELGADDA	AFVFGSGR	GHWRGSGY	FP	GW	1191
mkRyR3	1091	EVVTTGGDMRVG	1091	WARPGCRPD	IELGADDA	AFVFGSGR	GHWRGSGY	FP	GW	1191
mkRyR3	1191	SLGSLQIGRMN	1191	LGDSATFKFY	TCMGLQEG	FEFFAVNNR	DAVAMW	SK	RL	1291
mkRyR3	1191	SLGSLQIGRMN	1191	LGDSATFKFY	TCMGLQEG	FEFFAVNNR	DAVAMW	SK	RL	1291
mkRyR3	1191	SLGSLQIGRMN	1191	LGDSATFKFY	TCMGLQEG	FEFFAVNNR	DAVAMW	SK	RL	1291
mkRyR3	1191	SLGSLQIGRMN	1191	LGDSATFKFY	TCMGLQEG	FEFFAVNNR	DAVAMW	SK	RL	1291
mkRyR3	1291	MPVECHSSSHSP	1291	CLD...	SEAFQK	RMQGEIL	SH	TT	TC	1381
mkRyR3	1291	MPVECHSSSHSP	1291	CLD...	SEAFQK	RMQGEIL	SH	TT	TC	1381
mkRyR3	1291	MPVECHSSSHSP	1291	CLD...	SEAFQK	RMQGEIL	SH	TT	TC	1381
mkRyR3	1291	MPVECHSSSHSP	1291	CLD...	SEAFQK	RMQGEIL	SH	TT	TC	1381
mkRyR3	1381	LVVGGDILVAT	1381	SGSRSSNVLE	IG	CVLD	AT	GL	SFT	1481
mkRyR3	1381	LVVGGDILVAT	1381	SGSRSSNVLE	IG	CVLD	AT	GL	SFT	1481
mkRyR3	1381	LVVGGDILVAT	1381	SGSRSSNVLE	IG	CVLD	AT	GL	SFT	1481
mkRyR3	1381	LVVGGDILVAT	1381	SGSRSSNVLE	IG	CVLD	AT	GL	SFT	1481
mkRyR3	1481	RLDVQTI	1481	QPVLSRMP	SFLK	VE	TS	ER	HGW	1581
mkRyR3	1481	RLDVQTI	1481	QPVLSRMP	SFLK	VE	TS	ER	HGW	1581
mkRyR3	1481	RLDVQTI	1481	QPVLSRMP	SFLK	VE	TS	ER	HGW	1581
mkRyR3	1481	RLDVQTI	1481	QPVLSRMP	SFLK	VE	TS	ER	HGW	1581
mkRyR3	1581	LYAIDONKYL	1581	PALLASGFYDL	LI	SI	HL	NA	KER	1681
mkRyR3	1581	LYAIDONKYL	1581	PALLASGFYDL	LI	SI	HL	NA	KER	1681
mkRyR3	1581	LYAIDONKYL	1581	PALLASGFYDL	LI	SI	HL	NA	KER	1681
mkRyR3	1581	LYAIDONKYL	1581	PALLASGFYDL	LI	SI	HL	NA	KER	1681
mkRyR3	1681	ILTKKALSM	1681	TEAYSG	SAH	IRDP	VG	SG	VE	1781
mkRyR3	1681	ILTKKALSM	1681	TEAYSG	SAH	IRDP	VG	SG	VE	1781
mkRyR3	1681	ILTKKALSM	1681	TEAYSG	SAH	IRDP	VG	SG	VE	1781
mkRyR3	1681	ILTKKALSM	1681	TEAYSG	SAH	IRDP	VG	SG	VE	1781
mkRyR3	1781	DAKEAPVRL	1781	LLQTLAL	PSVKLQ	MC	EL	SYL	CD	1881
mkRyR3	1781	DAKEAPVRL	1781	LLQTLAL	PSVKLQ	MC	EL	SYL	CD	1881
mkRyR3	1781	DAKEAPVRL	1781	LLQTLAL	PSVKLQ	MC	EL	SYL	CD	1881
mkRyR3	1781	DAKEAPVRL	1781	LLQTLAL	PSVKLQ	MC	EL	SYL	CD	1881
mkRyR3	1881	ENCP	1881	PCPEE	IR	EL	YD	IF	ED	1981
mkRyR3	1881	ENCP	1881	PCPEE	IR	EL	YD	IF	ED	1981
mkRyR3	1881	ENCP	1881	PCPEE	IR	EL	YD	IF	ED	1981
mkRyR3	1881	ENCP	1881	PCPEE	IR	EL	YD	IF	ED	1981
mkRyR3	1981	SLLR	1981	QYDS	IG	ELL	QAL	K	RT	2081
mkRyR3	1981	SLLR	1981	QYDS	IG	ELL	QAL	K	RT	2081
mkRyR3	1981	SLLR	1981	QYDS	IG	ELL	QAL	K	RT	2081
mkRyR3	1981	SLLR	1981	QYDS	IG	ELL	QAL	K	RT	2081
mkRyR3	2081	QIAFPK	2081	WVASC	CRFL	CF	YCR	IR	SN	2181
mkRyR3	2081	QIAFPK	2081	WVASC	CRFL	CF	YCR	IR	SN	2181
mkRyR3	2081	QIAFPK	2081	WVASC	CRFL	CF	YCR	IR	SN	2181
mkRyR3	2081	QIAFPK	2081	WVASC	CRFL	CF	YCR	IR	SN	2181
mkRyR3	2181	PDVGNP	2181	IEG	ERYLS	FL	RA	FV	NS	2281
mkRyR3	2181	PDVGNP	2181	IEG	ERYLS	FL	RA	FV	NS	2281
mkRyR3	2181	PDVGNP	2181	IEG	ERYLS	FL	RA	FV	NS	2281
mkRyR3	2181	PDVGNP	2181	IEG	ERYLS	FL	RA	FV	NS	2281
mkRyR3	2281	AIMS	2281	FYSAL	IDL	LR	CA	PE	M	2381
mkRyR3	2281	AIMS	2281	FYSAL	IDL	LR	CA	PE	M	2381
mkRyR3	2281	AIMS	2281	FYSAL	IDL	LR	CA	PE	M	2381
mkRyR3	2281	AIMS	2281	FYSAL	IDL	LR	CA	PE	M	2381
mkRyR3	2381	LL	2381	EV	GL	FL	DL	RA	SL	2481
mkRyR3	2381	LL	2381	EV	GL	FL	DL	RA	SL	2481
mkRyR3	2381	LL	2381	EV	GL	FL	DL	RA	SL	2481
mkRyR3	2381	LL	2381	EV	GL	FL	DL	RA	SL	2481
mkRyR3	2481	QLLR	2481	RLV	FO	VP	Q	LN	EY	2581
mkRyR3	2481	QLLR	2481	RLV	FO	VP	Q	LN	EY	2581
mkRyR3	2481	QLLR	2481	RLV	FO	VP	Q	LN	EY	2581
mkRyR3	2481	QLLR	2481	RLV	FO	VP	Q	LN	EY	2581

Fig. 2. Amino acid sequence of the mink RyR3 (mkRyR3). The sequence, as deduced from the cDNA sequence, is aligned with those of the rabbit [9], chicken [27] and frog [28]. Amino acids are shown in single letter code. Identical amino acids or conservative substitutions are boxed. Gaps in the amino acid sequence are indicated by dashes. Alignment was obtained with the GCG sequence analysis software.

mRyR3	2582	ATLEKQISVDAGNFPKPPINTMNFSLPEKWEIVITKYAEHSHDKWACDSONGWKYGISLDENVKTHPLIPFFKTLTEKEKIYRWPAHRESLTKMLAVG	2681
rRyR3	2587	ATLEKQISVDAGNFPKPPINTMNFSLPEKWEIVITKYAEHSHDKWACDSONGWKYGISLDENVKTHPLIPFFKTLTEKEKIYRWPAHRESLTKMLAVG	2686
cRyR3	2586	STLEKQISVDAGNFPKPPINTMNFSLPEKWEIVITKYAEHSHDKWACDSONGWKYGISLDENVKTHPLIPFFKTLTEKEKIYRWPAHRESLTKMLAVG	2685
mRyR3	2587	ATLEKQISVDAGNFPKPPINTMNFSLPEKWEIVITKYAEHSHDKWACDSONGWKYGISLDENVKTHPLIPFFKTLTEKEKIYRWPAHRESLTKMLAVG	2686
mRyR3	2682	WSVETKELGEGALVQORENEKLRSVSSGASGNGSYSPAPDLNSNVLSRELOQMVEVMAENYHNIWAKKKKLESGQGGSHPLLVPYDTLTAKEKFRDRE	2780
rRyR3	2687	WTVETKELGEGALVQORENEKLRSVSSGASGNGSYSPAPDLNSNVLSRELOQMVEVMAENYHNIWAKKKKLESGQGGSHPLLVPYDTLTAKEKFRDRE	2785
cRyR3	2686	WSVETKELGEGALVQORENEKLRSVSSGASGNGSYSPAPDLNSNVLSRELOQMVEVMAENYHNIWAKKKKLESGQGGSHPLLVPYDTLTAKEKFRDRE	2783
mRyR3	2687	WSVETKELGEGALVQORENEKLRSVSSGASGNGSYSPAPDLNSNVLSRELOQMVEVMAENYHNIWAKKKKLESGQGGSHPLLVPYDTLTAKEKFRDRE	2785
mRyR3	2781	KAQDLFFFLQVNGIIVSRGKVMELDASSMEKRFAYFKLLKLLKYVDSAGFEIAHLEAVSSGKTEKSPHDOEIKFFAKVLLPLVDQYFTNHLLVFLSSP	2880
rRyR3	2786	KAQDLFFFLQVNGIIVSRGKVMELDASSMEKRFAYFKLLKLLKYVDSAGFEIAHLEAVSSGKTEKSPHDOEIKFFAKVLLPLVDQYFTNHLLVFLSSP	2885
cRyR3	2785	KAQDLFFFLQVNGIIVSRGKVMELDASSMEKRFAYFKLLKLLKYVDSAGFEIAHLEAVSSGKTEKSPHDOEIKFFAKVLLPLVDQYFTNHLLVFLSSP	2883
mRyR3	2786	KAQDLFFFLQVNGIIVSRGKVMELDASSMEKRFAYFKLLKLLKYVDSAGFEIAHLEAVSSGKTEKSPHDOEIKFFAKVLLPLVDQYFTNHLLVFLSSP	2885
mRyR3	2881	LKPLSSSGYASHKEKEMVASLFCFLAALVHRHISLFGSOSTTVMVSLCHILAQTLQDTRVMKSGSELVKAQRAFFENAAEOLKTESNKLKGFTHSRTO	2980
rRyR3	2886	LKPLSSSGYASHKEKEMVASLFCFLAALVHRHISLFGSOSTTVMVSLCHILAQTLQDTRVMKSGSELVKAQRAFFENAAEOLKTESNKLKGFTHSRTO	2985
cRyR3	2885	LKPLSSSGYASHKEKEMVASLFCFLAALVHRHISLFGSOSTTVMVSLCHILAQTLQDTRVMKSGSELVKAQRAFFENAAEOLKTESNKLKGFTHSRTO	2983
mRyR3	2886	LKPLSSSGYASHKEKEMVASLFCFLAALVHRHISLFGSOSTTVMVSLCHILAQTLQDTRVMKSGSELVKAQRAFFENAAEOLKTESNKLKGFTHSRTO	2985
mRyR3	2981	IKGVSONINNTYVALLPILTSIFEHVAOHQFVGLLLDQVQVSCYHILCSLSVLTQKNIYVERORPALGECASLAAAIIPVAFLEPLNRYNPLSVFNT	3080
rRyR3	2986	IKGVSONINNTYVALLPILTSIFEHVAOHQFVGLLLDQVQVSCYHILCSLSVLTQKNIYVERORPALGECASLAAAIIPVAFLEPLNRYNPLSVFNT	3085
cRyR3	2985	IKGVSONINNTYVALLPILTSIFEHVAOHQFVGLLLDQVQVSCYHILCSLSVLTQKNIYVERORPALGECASLAAAIIPVAFLEPLNRYNPLSVFNT	3083
mRyR3	2986	IKGVSONINNTYVALLPILTSIFEHVAOHQFVGLLLDQVQVSCYHILCSLSVLTQKNIYVERORPALGECASLAAAIIPVAFLEPLNRYNPLSVFNT	3085
mRyR3	3081	KTPREARSILGMPDTEEMCPDIPOLEGLMKETISDLAESGARYTEMPHIVLEIPLMCLNLSYVWWEHGPHELPSTGPWCYKVTSEHLSVILGNILKTIINN	3180
rRyR3	3086	KTPREARSILGMPDTEEMCPDIPOLEGLMKETISDLAESGARYTEMPHIVLEIPLMCLNLSYVWWEHGPHELPSTGPWCYKVTSEHLSVILGNILKTIINN	3185
cRyR3	3085	KTPREARSILGMPDTEEMCPDIPOLEGLMKETISDLAESGARYTEMPHIVLEIPLMCLNLSYVWWEHGPHELPSTGPWCYKVTSEHLSVILGNILKTIINN	3183
mRyR3	3086	KTPREARSILGMPDTEEMCPDIPOLEGLMKETISDLAESGARYTEMPHIVLEIPLMCLNLSYVWWEHGPHELPSTGPWCYKVTSEHLSVILGNILKTIINN	3185
mRyR3	3181	NLGIDEASWKKRIAYVAQPIISKARPDLKXSHFIPITLKKKKAVKTVQEEGLKADYKGTQEAELLILDEFALVLCADLYAFYFPMILRYVDNRRSNWLK	3280
rRyR3	3186	NLGIDEASWKKRIAYVAQPIISKARPDLKXSHFIPITLKKKKAVKTVQEEGLKADYKGTQEAELLILDEFALVLCADLYAFYFPMILRYVDNRRSNWLK	3285
cRyR3	3185	NLGIDEASWKKRIAYVAQPIISKARPDLKXSHFIPITLKKKKAVKTVQEEGLKADYKGTQEAELLILDEFALVLCADLYAFYFPMILRYVDNRRSNWLK	3283
mRyR3	3186	NLGIDEASWKKRIAYVAQPIISKARPDLKXSHFIPITLKKKKAVKTVQEEGLKADYKGTQEAELLILDEFALVLCADLYAFYFPMILRYVDNRRSNWLK	3285
mRyR3	3281	SPSADSDOLFMAVAEYFVLWCKSHNFKREONFVIGNEINNLAFITGDSKSKMSKAMVGGGGGGGAKKTKRRDGLYSIOTSLIYAAALKKMLPIGLNMC	3375
rRyR3	3286	SPSADSDOLFMAVAEYFVLWCKSHNFKREONFVIGNEINNLAFITGDSKSKMSKAMVGGGGGGGAKKTKRRDGLYSIOTSLIYAAALKKMLPIGLNMC	3380
cRyR3	3285	SPSADSDOLFMAVAEYFVLWCKSHNFKREONFVIGNEINNLAFITGDSKSKMSKAMVGGGGGGGAKKTKRRDGLYSIOTSLIYAAALKKMLPIGLNMC	3383
mRyR3	3286	SPSADSDOLFMAVAEYFVLWCKSHNFKREONFVIGNEINNLAFITGDSKSKMSKAMVGGGGGGGAKKTKRRDGLYSIOTSLIYAAALKKMLPIGLNMC	3385
mRyR3	3376	TPGGOELISLAKSRYSYADTDEEVKEHLRNNLHLOEKSDPAVWQLNLKYLKSDSEEPNPEKTVERVORISAAVFLHQQVEOPLRSKAAVWHKLLSK	3475
rRyR3	3381	TPGGOELISLAKSRYSYADTDEEVKEHLRNNLHLOEKSDPAVWQLNLKYLKSDSEEPNPEKTVERVORISAAVFLHQQVEOPLRSKAAVWHKLLSK	3480
cRyR3	3380	TPGGOELISLAKSRYSYADTDEEVKEHLRNNLHLOEKSDPAVWQLNLKYLKSDSEEPNPEKTVERVORISAAVFLHQQVEOPLRSKAAVWHKLLSK	3478
mRyR3	3381	TPGGOELISLAKSRYSYADTDEEVKEHLRNNLHLOEKSDPAVWQLNLKYLKSDSEEPNPEKTVERVORISAAVFLHQQVEOPLRSKAAVWHKLLSK	3480
mRyR3	3476	RKRAVACFAMAPLYNLPRHRSINFLHQQVEOPLRSKAAVWHKLLSK	3574
rRyR3	3481	RKRAVACFAMAPLYNLPRHRSINFLHQQVEOPLRSKAAVWHKLLSK	3579
cRyR3	3480	RKRAVACFAMAPLYNLPRHRSINFLHQQVEOPLRSKAAVWHKLLSK	3578
mRyR3	3481	RKRAVACFAMAPLYNLPRHRSINFLHQQVEOPLRSKAAVWHKLLSK	3579
mRyR3	3575	SSMMAKSCSGGDEEEEDDEKKTFFEEKEMKOKTLYQOARHERGAAEMVLQMSISAKSGGEMPMVETLKLQIAILNGGNTVQQKMLDYLKKEKDAOFF	3674
rRyR3	3580	SSMMAKSCSGGDEEEEDDEKKTFFEEKEMKOKTLYQOARHERGAAEMVLQMSISAKSGGEMPMVETLKLQIAILNGGNTVQQKMLDYLKKEKDAOFF	3679
cRyR3	3579	SSMMAKSCSGGDEEEEDDEKKTFFEEKEMKOKTLYQOARHERGAAEMVLQMSISAKSGGEMPMVETLKLQIAILNGGNTVQQKMLDYLKKEKDAOFF	3678
mRyR3	3580	SSMMAKSCSGGDEEEEDDEKKTFFEEKEMKOKTLYQOARHERGAAEMVLQMSISAKSGGEMPMVETLKLQIAILNGGNTVQQKMLDYLKKEKDAOFF	3679
mRyR3	3675	QSLSGMLMOSCVLDLNAFERONKKAEGLMVTEEGTILVIRERGEKVLONDEFTDLFRFOLLCEGHNSDFONFLATQMNTTNNVISTVDYLLALQES	3768
rRyR3	3680	QSLSGMLMOSCVLDLNAFERONKKAEGLMVTEEGTILVIRERGEKVLONDEFTDLFRFOLLCEGHNSDFONFLATQMNTTNNVISTVDYLLALQES	3773
cRyR3	3679	QSLSGMLMOSCVLDLNAFERONKKAEGLMVTEEGTILVIRERGEKVLONDEFTDLFRFOLLCEGHNSDFONFLATQMNTTNNVISTVDYLLALQES	3772
mRyR3	3680	QSLSGMLMOSCVLDLNAFERONKKAEGLMVTEEGTILVIRERGEKVLONDEFTDLFRFOLLCEGHNSDFONFLATQMNTTNNVISTVDYLLALQES	3773
mRyR3	3769	ISDFWYVYSGKDIIDESGQHNSKALAYTKOIFNSLTETIOGPGICGNQSLAHSRLWDVAVGFLHYFANMOMKLSQDSOIELLKELLQDLMVVMVLS	3868
rRyR3	3774	ISDFWYVYSGKDIIDESGQHNSKALAYTKOIFNSLTETIOGPGICGNQSLAHSRLWDVAVGFLHYFANMOMKLSQDSOIELLKELLQDLMVVMVLS	3873
cRyR3	3773	ISDFWYVYSGKDIIDESGQHNSKALAYTKOIFNSLTETIOGPGICGNQSLAHSRLWDVAVGFLHYFANMOMKLSQDSOIELLKELLQDLMVVMVLS	3872
mRyR3	3774	ISDFWYVYSGKDIIDESGQHNSKALAYTKOIFNSLTETIOGPGICGNQSLAHSRLWDVAVGFLHYFANMOMKLSQDSOIELLKELLQDLMVVMVLS	3873
mRyR3	3869	ILEGNVYVNGTIGKQVMDTLVSSSNVEMILKFFDMFLKLDLSSDTFFKEDPDGQKIISSKKEFOKAMEGQKQYTOSEIDFLSCAEADENOMFNYIOFV	3968
rRyR3	3874	ILEGNVYVNGTIGKQVMDTLVSSSNVEMILKFFDMFLKLDLSSDTFFKEDPDGQKIISSKKEFOKAMEGQKQYTOSEIDFLSCAEADENOMFNYIOFV	3973
cRyR3	3873	ILEGNVYVNGTIGKQVMDTLVSSSNVEMILKFFDMFLKLDLSSDTFFKEDPDGQKIISSKKEFOKAMEGQKQYTOSEIDFLSCAEADENOMFNYIOFV	3972
mRyR3	3874	ILEGNVYVNGTIGKQVMDTLVSSSNVEMILKFFDMFLKLDLSSDTFFKEDPDGQKIISSKKEFOKAMEGQKQYTOSEIDFLSCAEADENOMFNYIOFV	3973
mRyR3	3969	DRFHEPAXDIGFNVAULTNLSEHMPNDSRLKCLDPA-DSVNLNYPEPYLGRIEMGQAKKIERVYFEISSESRTOWEKPOVKESKROPIFOVNVGEGE	4067
rRyR3	3974	DRFHEPAXDIGFNVAULTNLSEHMPNDSRLKCLDPA-DSVNLNYPEPYLGRIEMGQAKKIERVYFEISSESRTOWEKPOVKESKROPIFOVNVGEGE	4072
cRyR3	3973	DRFHEPAXDIGFNVAULTNLSEHMPNDSRLKCLDPA-DSVNLNYPEPYLGRIEMGQAKKIERVYFEISSESRTOWEKPOVKESKROPIFOVNVGEGE	4071
mRyR3	3974	DRFHEPAXDIGFNVAULTNLSEHMPNDSRLKCLDPA-DSVNLNYPEPYLGRIEMGQAKKIERVYFEISSESRTOWEKPOVKESKROPIFOVNVGEGE	4072
mRyR3	4068	EXMELFVNFCEDTIFEMOLASOISESDADPDEEEEDDESSVYVLEIINGEEEDKSFESAFAMACAKRNITNLLKATLKNLRKOYAKVKKMTVK	4167
rRyR3	4073	EXMELFVNFCEDTIFEMOLASOISESDADPDEEEEDDESSVYVLEIINGEEEDKSFESAFAMACAKRNITNLLKATLKNLRKOYAKVKKMTVK	4172
cRyR3	4072	EXMELFVNFCEDTIFEMOLASOISESDADPDEEEEDDESSVYVLEIINGEEEDKSFESAFAMACAKRNITNLLKATLKNLRKOYAKVKKMTVK	4171
mRyR3	4073	EXMELFVNFCEDTIFEMOLASOISESDADPDEEEEDDESSVYVLEIINGEEEDKSFESAFAMACAKRNITNLLKATLKNLRKOYAKVKKMTVK	4172
mRyR3	4168	ELVKYFSSFFWMLFVGLFOLFFFTIVGGIFOLWSTVFGGGLVEGAKNIRVTKILGOMPPTQFGIHDADAEAEVTEGVPVPLINPVKGEKQDADIM	4267
rRyR3	4173	ELVKYFSSFFWMLFVGLFOLFFFTIVGGIFOLWSTVFGGGLVEGAKNIRVTKILGOMPPTQFGIHDADAEAEVTEGVPVPLINPVKGEKQDADIM	4272
cRyR3	4172	ELVKYFSSFFWMLFVGLFOLFFFTIVGGIFOLWSTVFGGGLVEGAKNIRVTKILGOMPPTQFGIHDADAEAEVTEGVPVPLINPVKGEKQDADIM	4271
mRyR3	4173	ELVKYFSSFFWMLFVGLFOLFFFTIVGGIFOLWSTVFGGGLVEGAKNIRVTKILGOMPPTQFGIHDADAEAEVTEGVPVPLINPVKGEKQDADIM	4272
mRyR3	4268	SOLFGLHPPKKEGGVXKHPDEGLDGLSEIIGDEPPLESTVVRKKAQAAEMKAAHEAGKVEPERTDLEDGEREDJAKEEEDGAEALWADYTKKKKRRRG	4367
rRyR3	4273	SOLFGLHPPKKEGGVXKHPDEGLDGLSEIIGDEPPLESTVVRKKAQAAEMKAAHEAGKVEPERTDLEDGEREDJAKEEEDGAEALWADYTKKKKRRRG	4372
cRyR3	4272	SOLFGLHPPKKEGGVXKHPDEGLDGLSEIIGDEPPLESTVVRKKAQAAEMKAAHEAGKVEPERTDLEDGEREDJAKEEEDGAEALWADYTKKKKRRRG	4371
mRyR3	4273	SOLFGLHPPKKEGGVXKHPDEGLDGLSEIIGDEPPLESTVVRKKAQAAEMKAAHEAGKVEPERTDLEDGEREDJAKEEEDGAEALWADYTKKKKRRRG	4372
mRyR3	4368	QXVEXPEAFMANFVKGLEIYOTKLLHLYARNFYNLRFALFVAFAINFILLFYKYTTEPVEEETEDVANLWNSPIDEED-EEAMVFFVLOESTGYMAPA	4465
rRyR3	4373	QXVEXPEAFMANFVKGLEIYOTKLLHLYARNFYNLRFALFVAFAINFILLFYKYTTEPVEEETEDVANLWNSPIDEED-EEAMVFFVLOESTGYMAPA	4470
cRyR3	4372	QXVEXPEAFMANFVKGLEIYOTKLLHLYARNFYNLRFALFVAFAINFILLFYKYTTEPVEEETEDVANLWNSPIDEED-EEAMVFFVLOESTGYMAPA	4469
mRyR3	4373	QXVEXPEAFMANFVKGLEIYOTKLLHLYARNFYNLRFALFVAFAINFILLFYKYTTEPVEEETEDVANLWNSPIDEED-EEAMVFFVLOESTGYMAPA	4470
mRyR3	4466	LRALATIHIVISLVCVGVYGLKVLVVFVFKREKEIARKLEFDGLYITEQPSDDIKGQWDLVINTPSPFNPNYWDKFKVRKVKYKQDLYGAERIELLG	4565
rRyR3	4471	LRALATIHIVISLVCVGVYGLKVLVVFVFKREKEIARKLEFDGLYITEQPSDDIKGQWDLVINTPSPFNPNYWDKFKVRKVKYKQDLYGAERIELLG	4570
cRyR3	4470	LRALATIHIVISLVCVGVYGLKVLVVFVFKREKEIARKLEFDGLYITEQPSDDIKGQWDLVINTPSPFNPNYWDKFKVRKVKYKQDLYGAERIELLG	4569
mRyR3	4471	LRALATIHIVISLVCVGVYGLKVLVVFVFKREKEIARKLEFDGLYITEQPSDDIKGQWDLVINTPSPFNPNYWDKFKVRKVKYKQDLYGAERIELLG	4570
mRyR3	4566	LQKNAIDFSPVEASKAEEASLVSWLSSIDMKYHIWKLGVYFTDNSFLYLAWYTTMSVLGHYNNFFFAAHLDIAMGFKTLRTILSSVTHNQKQLVTVGL	4665
rRyR3	4571	LQKNAIDFSPVEASKAEEASLVSWLSSIDMKYHIWKLGVYFTDNSFLYLAWYTTMSVLGHYNNFFFAAHLDIAMGFKTLRTILSSVTHNQKQLVTVGL	4670
cRyR3	4570	LQKNAIDFSPVEASKAEEASLVSWLSSIDMKYHIWKLGVYFTDNSFLYLAWYTTMSVLGHYNNFFFAAHLDIAMGFKTLRTILSSVTHNQKQLVTVGL	4669
mRyR3	4571	LQKNAIDFSPVEASKAEEASLVSWLSSIDMKYHIWKLGVYFTDNSFLYLAWYTTMSVLGHYNNFFFAAHLDIAMGFKTLRTILSSVTHNQKQLVTVGL	4670
mRyR3	4666	LAVVYVLYTVYAFNFFRKFYKNSKEDDDPDMKCDMMTCYLFHMYGVGRAGGIGDEEDPAGDPYEMRYIVFDITFFFFVIVILLAIQGLIIDAFGEL	4765
rRyR3	4671	LAVVYVLYTVYAFNFFRKFYKNSKEDDDPDMKCDMMTCYLFHMYGVGRAGGIGDEEDPAGDPYEMRYIVFDITFFFFVIVILLAIQGLIIDAFGEL	4770
cRyR3	4670	LAVVYVLYTVYAFNFFRKFYKNSKEDDDPDMKCDMMTCYLFHMYGVGRAGGIGDEEDPAGDPYEMRYIVFDITFFFFVIVILLAIQGLIIDAFGEL	4769
mRyR3	4671	LAVVYVLYTVYAFNFFRKFYKNSKEDDDPDMKCDMMTCYLFHMYGVGRAGGIGDEEDPAGDPYEMRYIVFDITFFFFVIVILLAIQGLIIDAFGEL	4770
mRyR3	4766	RODQEOVRDMETKCFICGIGNDYFDTTPHGFETHLOEHNILANYLLFMYLINDKDETEHTGOESYVWKMVQERCDWFFPAGDGFRAQYEDOLG	4859
rRyR3	4771	RODQEOVRDMETKCFICGIGNDYFDTTPHGFETHLOEHNILANYLLFMYLINDKDETEHTGOESYVWKMVQERCDWFFPAGDGFRAQYEDOLG	4864
cRyR3	4770	RODQEOVRDMETKCFICGIGNDYFDTTPHGFETHLOEHNILANYLLFMYLINDKDETEHTGOESYVWKMVQERCDWFFPAGDGFRAQYEDOLG	4859
mRyR3	4771	RODQEOVRDMETKCFICGIGNDYFDTTPHGFETHLOEHNILANYLLFMYLINDKDETEHTGOESYVWKMVQERCDWFFPAGDGFRAQYEDOLG	4864

signed specific primers that should amplify a cDNA fragment of 355 bp, should the 15 bp insertion be present [ASI(+)], and a fragment of 340 bp in absence of the insertion [ASI(-)]. Other primers were designed to amplify a fragment of 295 bp in the presence of the 18 bp insertion [ASII(+)] and of 277 bp in its absence [ASII(-)]. The fragments amplified with these primers on cDNA retrotranscribed from 10 µg of RNA prepared from mink brain were cloned into the pBlue-script plasmid and sequenced. The clones identified differed

for the presence or the absence of two insertion of 15 or 18 bp. The amino acid sequences encoded by the 15 and 18 bp insertion sequences are shown in Fig. 3.

An accurate comparative analysis of the mink RyR3 amino acid sequence pointed out a slight difference with respect to other RyRs in the region spanning amino acids 3495–3527 (Fig. 2). To verify the possibility that this could represent a further site of alternative splicing, we designed a set of primers that would amplify a cDNA fragment of 311 bp encompass-

ASI (-)	MSK - - - - SGGQDQERK
ASI (+)	MSK A M Q V K SGGQDQERK
ASII (-)	TEEGT - - - - REKVLQNDEFT
ASII (+)	TEEGT L I V R E R GEKVLQNDEFT
ASIII exon A	KINNFFLSTFORVWLEKVNKTQY.DRLIPILM
ASIII exon B	RSI.NLPLHGYQRFWIE..TEEYSFEELKLVQDLA

Fig. 3. Amino acid sequence of ASI and ASII insertion and of the two mutually exclusive exons A and B in mink RyR3 cDNA.

ing this region. The amplified products from mink brain cDNA were cloned in *EcoRV/SmaI* sites of pBluescript and sequenced. Sequence analysis of these clones led to the identification of a new exon, different from that previously identified in CCL64, but similar to that of other RyR3 sequences (Fig. 3). This region represents, therefore, a third site of alternative splicing (ASIII). These two exons are referred to as exons A and B in Figs. 3 and 4c and in the text. Comparison between the amino acid sequences of exons A and B revealed that they share 64% homology (Fig. 3).

The genomic organization of the region of the RyR3 gene containing exons A and B has been defined by PCR amplification of genomic DNA prepared from CCL64 cells. The exon/intron boundaries have been established. Exon A has been found to be located upstream of exon B, with an intronic sequence of about 1000 bp separating them. An intronic sequence of about 2000 bp is located between exon A and the upstream exon in the RyR3 genomic sequence and an intronic sequence of about 1500 bp is located between exon B and the downstream exon (data not shown).

3.4. Expression pattern of the alternatively spliced RyR3 transcripts in different mink tissues

In order to define the tissue distribution of the alternatively spliced RyR3 mRNAs we performed PCR amplification of cDNA derived from RNA extracted from different mink tissues, using the previously described pairs of primers specific for alternative splicing sites I and II as shown in Fig. 4. In order to confirm that the amplified fragments corresponded to the expected regions of the alternative splicing sites I and II, they were digested with specific restriction enzymes. The *MseI* digestion of the PCR products containing alternative splicing site I yields two fragments of 223 bp in the presence of the 15 bp insertion [ASI(+)] and of 208 bp without the insertion [ASI(-)] (Fig. 4a). Two fragments of 115 and 17 bp are common to both mRNA species (not shown). Both the 223 and 208 bp fragments can be detected in all tissues analyzed as well as in CCL64 cells, indicating that both alternatively spliced mRNAs are present (Fig. 4a).

Similarly, amplified DNA fragments corresponding to alternative splicing site II were digested with *NaeI* restriction enzyme, which gives two fragments of 233 bp for the mRNA containing the 18 bp insertion [ASII(+)] and of 215 bp in absence of it [ASII(-)] (Fig. 4b). A fragment of 62 bp is common to the two alternatively spliced forms (not shown). As shown in Fig. 4b both forms are present in cerebellum, muscle, ovary and CCL64 cells. The fragment corresponding to the mRNA containing the 18 bp insertion is present in

brain and diaphragm, while only the fragment corresponding to the mRNA without the insertion is detected in ileum.

The tissue distribution of the third splicing site (ASIII) was defined using specific primers for exons A and B (Fig. 4c). The mRNA containing exon A was detected in all tissues analyzed as well as in CCL64 cells, while the mRNA containing exon B is present in brain, cerebellum, skeletal muscle, diaphragm and ileum, while it is not detected in ovary and CCL64.

4. Discussion

In the present study we have reported the complete sequence of the RyR3 cDNA cloned from the mink lung epithelial cell line CCL64. The deduced amino acid sequence shows close similarity with those of other RyRs, although the sequence corresponding to amino acids 1116–1140 differs from the corresponding region of rabbit RyR3, probably due to an out-of-frame in the reported rabbit nucleotide sequence (Fig. 2).

In our first report on mink RyR3, we isolated three clones that all lacked the region encoding the transmembrane sequence M5 [7].

Further analysis revealed that about 7 out of 10 of the RyR3 mRNA expressed in CCL64 lack this region and that similar transcripts can be detected also in some mink tissues, although at lower levels (data not shown). While performing these studies, we also observed the existence of RyR3 anomalous transcripts with some exons skipped (V. Sorrentino, data not shown). As a consequence of this process, termination codons were introduced, so that these mRNAs would, if translated, encode shorter proteins unless decoded by a mechanism similar to programmed ribosomal frameshifting [19]. A similar finding has been reported by Chen et al. for rabbit RyR3 mRNA [20].

We report here the existence of three transcripts of RyR3 mRNA which differ for the insertion of short exons in two cases, or in the alternative usage of one out of two possible exons, as a consequence of classic alternative splicing process. The first two alternative splicing sites consist of insertion of 5 amino acids (Ala-Met-Gln-Val-Lys) after residue 3335 and of 6 amino acids (Leu-Ile-Val-Arg-Glu-Arg) after residue 3710. Similar alternative splicing regions have been observed in human, rabbit and mouse RyR1 cDNA [13,14,21].

The third splicing site is defined by a mutually exclusive splicing mechanism, in which exon A or exon B can be alternatively included in the mature RyR3 mRNA. The two exons share 64% homology, suggesting that they may have been derived from the duplication of an ancestral element. Unlike alternative splicing sites I and II, alternative splicing site III has not been found yet in either RyR1 or RyR2.

The tissue distribution of these alternatively spliced RyR3 mRNAs has revealed that, while no differential expression has been observed for the two alternative splicing site I variants, splice variants of the alternative splicing sites II and III present a specific differential pattern of expression in the tissues analyzed (Fig. 4). Futatsugi et al. have reported a differential expression pattern of the corresponding regions of the alternative splicing sites I and II in RyR1 transcripts, in mouse skeletal muscle, heart, cerebellum and cerebrum [14].

The alternative splicing sites identified in RyR3, as well as those observed in RyR1 and RyR2 mRNA, are located in a limited region upstream of the transmembrane domains, com-

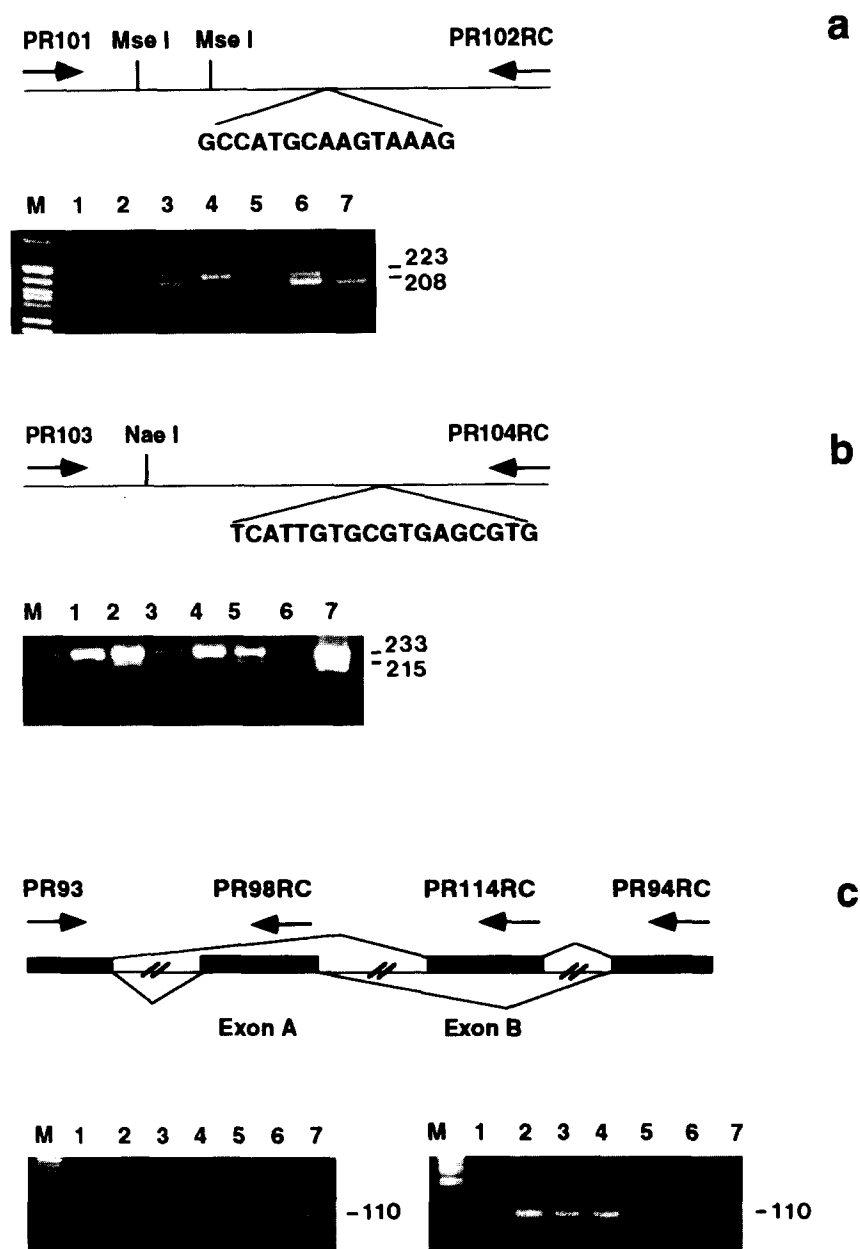


Fig. 4. PCR analysis of splicing patterns of ASI, ASII and ASIII in different mink tissues. cDNA from mink brain (1), cerebellum (2), muscle (3), diaphragm (4), ovary (5), ileum (6) and CCL64 (7) were amplified with primers PR101 and PR102RC (a), PR103 and PR104RC (b) and PR93 and PR98RC (c, exon A) and PR93 and PR114RC (c, exon B). PCR fragments corresponding to ASI and ASII were digested with *Mse*I and *Nae*I, respectively. The digestion products and the PCR fragments corresponding to ASIII were analyzed on 3% NuSieve-0.5% agarose gel. The two fragments of 223 and 208 bp, corresponding to ASI(+) and ASI(-), respectively, are shown in a. The two fragments of 233 and 215 bp corresponding to ASII(+) and ASII(-), respectively, are shown in b. Two fragments of 110 bp relative to ASIII exon A and ASIII exon B were amplified using specific primers (c).

monly indicated as modulatory region, which contains many potential regulatory and modification sites. Alternative splicing site II is localized in a region with a sequence highly conserved between RyRs and the InsP_3 type 1 receptor [8]. It is also noteworthy that the alternative splicing site III in the mink RyR3 mRNA is adjacent to the sequence coding for one of the three recently identified CaM binding sites (CaM2), present in all RyR isoforms (3474–3492 in the rabbit RyR3) [22]. CaM has been shown to affect the calcium release mediated by RyRs in a very complex fashion. Tripathy et al. have demonstrated that calmodulin may have an opposite effect on RyR1 [23]. At low calcium concentration (100–150

nM) CaM binds to the receptor, keeping it in an activated form. During E-C coupling, however, Ca^{2+} binds to CaM and the calcium-bound form of CaM seems to inactivate the channel, preventing further calcium release. These observations suggest that calmodulin may play an important role in modulating the activity of RyR1 in skeletal muscle contributing to the opening and closure of the channel. The possibility that different combinations of the alternatively spliced variants to form homo- and/or heterotetrameric channels may result in further heterogeneity in the calcium release properties of RyRs. This consideration is further supported by the characterization of the functional properties of the proteins encoded

by alternatively spliced transcripts of the InsP_3 receptor type 1 [11,24,25].

In conclusion, the data reported in the present work for RyR3 supply further evidence for the existence of alternatively spliced variants in the ryanodine receptor family. The localization of splicing sites in the putative modulatory region of the receptor sequence suggests that this mechanism may affect the activity of the channel, resulting in the existence of functionally distinguishable channels. An increasing number of reports indicate that intracellular calcium signalling is a very complex mechanism with multiple levels of regulation. It is commonly accepted that calcium signalling is the result of a combination of spatial-temporal events, in which the increase of $[\text{Ca}^{2+}]_i$ frequently appears as repetitive calcium waves or spikes that originate from discrete regions and propagate inside the cell in a regenerative manner [1,2]. A combination of factors can determine the generation and regulation of these complex calcium signals. The generation of spikes and waves inside the cell can be explained by the fact that both InsP_3 receptors and RyRs display the phenomenon of calcium-induced calcium release, in which calcium exerts a stimulatory effect on these receptors, priming a positive feedback mechanism [26]. In addition, it would be expected that the co-ordination of calcium release events to produce a specific calcium response may require the action of receptors with different sensitivity to calcium and/or other intracellular modulators [1,2]. Different molecular mechanisms may be responsible for the generation of functionally different channels at both the transcriptional and post-translational levels. The cell-specific co-expression of channels derived from alternative splicing events may contribute to such a process. The future characterization of the functional properties of the proteins encoded by the alternatively spliced transcripts would provide further insight into their role in calcium signalling mechanisms.

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