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Tissue-specific and developmentally regulated alternative splicing in mouse skeletal muscle ryanodine receptor mRNA

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The ryanodine receptor is a channel for Ca^{2+} release from intracellular stores. By PCR analysis, we identified two alternatively spliced regions in mRNA of the mouse skeletal muscle ryanodine receptor (sRyR). The splice variants were characterized by the presence or absence of 15 bp (ASI) and 18 bp (ASII) exons. The exclusion of these exons results in the absence of the regions corresponding to Ala^{3481}–Gln^{3485} and Val^{3865}–Asn^{3870}, respectively, of rabbit sRyR; these amino acid sequences exist in the modulatory region, where sites for phosphorylation and binding of Ca^{2+}, calmodulin and ATP are postulated to be. We also detected sRyR in brain and heart as well as in skeletal muscle, and the splicing patterns were found to be tissue-specific. Only the ASII-lacking isoform was detected in heart, whereas in other tissues the ASII-containing isoform was predominant. The splicing patterns were also found to change during development. In skeletal muscle, the ASI-containing isoform increased gradually from embryo to adult. The ASII-lacking isoform abruptly increased upon birth, but the ASII-containing isoform increased steadily afterwards. In cerebrum, the ratio of the ASII-containing isoform to the ASII-lacking one increased abruptly during embryonic days 14 and 18. These findings suggest that the alternative splicing of ASI and ASII, by affecting the modulatory region, generates functionally different sRyR isoforms in a tissue-specific and developmentally regulated manner.

INTRODUCTION

Depolarization of the plasma membrane of muscle cells leads to release of Ca^{2+} from the sarcoplasmic reticulum. The released Ca^{2+} plays a central role in the excitation–contraction coupling of muscle. Influx of Ca^{2+} into the cytoplasm is necessary for Ca^{2+} release in cardiac muscle (Ca^{2+}-induced Ca^{2+} release) (Nabauer et al., 1989), whereas depolarization of the plasma membrane, without Ca^{2+} influx, is sufficient in skeletal muscle (depolarization-induced Ca^{2+} release) (Rios and Brum, 1987). The ryanodine receptor (RyR), to which a plant alkaloid ryanodine specifically binds, is a major channel for calcium release from intracellular stores in cardiac and skeletal muscles and mediates Ca^{2+}-induced Ca^{2+} release and depolarization-induced Ca^{2+} release (Fleischer and Inui, 1989). It exists as a homotetramer; each subunit is about 500 kDa. Recent studies showed that RyR is also present in brain and smooth muscle, suggesting a role for RyR in Ca^{2+} mobilization in these tissues (Herrmann-Frank et al., 1991; Henzi and MacDermott, 1992; McPherson and Campbell, 1993a).

Three subtypes of RyRs are known to be encoded by different genes: the skeletal muscle type (sRyR) (Takeshima et al., 1989; Zorzato et al., 1990), the cardiac muscle type (cRyR) (Otsu et al., 1990; Nakai et al., 1990) and a third type called the brain type (bRyR) (Giannini et al., 1992; Hakamata et al., 1992). They show about 70% homology with each other and have common structural features: the large N-terminal domain comprising the foot region and the transmembrane domain near the C-terminus which is considered to form a channel. Between the N-terminal and transmembrane domains in sRyR and cRyR, there is a modulatory region where phosphorylation, and the binding of Ca^{2+}, ATP and calmodulin possibly modulate the function (see Figure 1). The three subtypes show different tissue distribution. cRyR is the major subtype in cardiac muscle. It is the major subtype also in brain and is present in various neurons (Kuwajima et al., 1992; Nakanishi et al., 1992; McPherson and Campbell, 1993b; Furuichi et al., 1994). sRyR is the major subtype in skeletal muscle and is present also in cerebellum, hippocampus and cerebral cortex (Kuwajima et al., 1992; Furuichi et al., 1994). bRyR is expressed in aorta and some parts of the brain (Hakamata et al., 1992). Although the difference in function among these three subtypes is unclear, several lines of evidence suggest that each of them has different properties. Since depolarization-induced Ca^{2+} release occurs in skeletal muscle and Ca^{2+}-induced Ca^{2+} release occurs in cardiac muscle, sRyR may mediate the former reaction while cRyR may mediate the latter. Recent studies suggest that cyclic ADP-ribose stimulates Ca^{2+} release through RyR in cardiac muscle and brain but not in skeletal muscle (Meszaros et al., 1993; White et al., 1993). Possibly, cRyR and/or bRyR but not sRyR is activated by cyclic ADP-ribose.

In addition to the above three subtypes, further heterogeneity of RyRs may result from alternative splicing of mRNA. In type I Ins(1,4,5)P_3 receptor (IP_3R), which is another channel for Ca^{2+} release and has structural features similar to those of RyR, alternative splicing in the N-terminal and modulatory region generates molecular heterogeneity (Nakagawa et al., 1991; Schell et al., 1993). In the present study, we examined the heterogeneity of mouse sRyR mRNA by the PCR technique and found two alternatively spliced regions localized in the modulatory region. We investigated the splicing pattern further in various tissues at various developmental stages. The results show that the alterna-

Abbreviations used: RyR, ryanodine receptor; sRyR, skeletal muscle ryanodine receptor; cRyR, cardiac muscle ryanodine receptor; bRyR, brain ryanodine receptor; IP_3R, Ins(1,4,5)P_3 receptor.

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tive splicings generate sRyR isoforms not only in a tissue-specific manner but also in a developmentally regulated manner.

EXPERIMENTAL

RNA preparation and cDNA synthesis

ICR mice were anaesthetized and killed by decapitation, and skeletal muscle of hind limb, heart, cerebrum and cerebellum were dissected. The total RNA of the adult tissues was isolated by the single-step acid guanidinium thiocyanate phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) and poly(A)$^{+}$ RNA was purified with Oligotex-dT30 (Takara Shuzo). Poly(A)$^{+}$ RNA of the tissues, from embryonic day 14 (E14) to postnatal day 21 (P21), was prepared with a Micro-Fast Track mRNA Isolation Kit (Invitrogen). Oligo(dT15)-primed synthesis of the cDNA first strand was carried out with Moloney murine leukaemia virus reverse transcriptase. The yield was calculated from the amount of [α-^{32}P]dCTP incorporated.

PCR-amplification of cDNA

PCR primers for the amplification of sRyR cDNA were designed on the basis of the cDNA sequence of rabbit sRyR, because molecular cloning of the mouse counterpart has not been reported. By comparison of the cDNA sequences of rabbit sRyR and cRyR, the primers were designed so as to amplify the cDNA of sRyR but not that of cRyR. For PCR amplification, 30–40 cycles of reaction consisting of the following steps were performed: denaturation at 94 °C for 1 min, annealing at 50–58 °C for 2 min, and elongation by AmpliTaq DNA polymerase at 74 °C for 3 min. The PCR products were subcloned into TA plasmids (Invitrogen) and the DNA sequences were determined by the dideoxy chain termination method. Direct DNA sequencing was also performed with the double-stranded DNA cycle sequencing system (Bethesda Research Laboratories). From these sequences, amplified products were confirmed to be derived from the sRyR cDNA.

PCR of genomic DNA regions containing ASI and ASII

ICR mouse genomic DNA was prepared from liver. Liver was homogenized with a Teflon Potter homogenizer in 10 mM Tris/HCl (pH 8.0) and 100 mM EDTA, and incubated at 37 °C for 1 h in the same buffer to which 0.5 % sarcosyl and 50 μg/ml RNAase A were added. The mixture was treated with 100 μg/ml of proteinase K at 60 °C overnight. Genomic DNA in the mixture was extracted with phenol, phenol/chloroform and diethyl ether. For PCR amplification of genomic DNA fragments that span ASI and ASII, the following primers were used: the sense primer S75, 5′-GACAATAAGAGCAAATGG-3′, and the antisense primer S72, 5′-CTTGGTGCGTTCCTGATATG-3′, for ASI; and the sense primer S79, 5′-CCTGGACCTCAATGCCTTCG-3′, and the antisense primer S74, 5′-GGTCCTGTGTGAACTCGTCA-3′, for ASII. S75, S72, S79 and S74 were designed on the basis of the mouse sRyR cDNA sequence, which we elucidated, and correspond to nucleotides 10548–10567, 10597–10616, 11654–11673 and 11762–11781 of the cDNA sequence of rabbit sRyR, respectively. Annealing temperatures in the PCR were 53 °C (S75 and S72) and 58 °C (S79 and S74), and 35 cycles of amplification were performed.

PCR-analysis of splicing pattern

The PCR primers S75 and S72, described above, were used for the reverse transcription-PCR analysis of the splicing pattern at ASI. For the analysis at ASII, the sense primer S77, 5′-CGAGAGGCAGAACAAGGCAG-3′, and the antisense primer S74 were used. S77 was derived from the cDNA sequence of mouse sRyR and corresponds to 11672–11691 of the cDNA of rabbit sRyR. We carried out 40 cycles of PCR using from 1 pg to 5 ng of cDNA as a template. Annealing temperatures were 53 °C (S75 and S72) and 58 °C (S77 and S74). The products were analysed by electrophoresis on 8 % polyacrylamide gel. Each primer set generated two fragments of different length according to the inclusion and the exclusion of ASI or ASII in the cDNA. The ratios of these two fragments were compared with those of the two fragments amplified from the standard templates by PCR, and the ratios ASI(+):ASI(−) and ASII(+):ASII(−) in template cDNA were estimated. The standard templates are mixtures of two plasmids into which cDNA fragments containing and lacking ASI or ASII were cloned; these plasmids were mixed at fixed molar ratios ranging from 30:1 to 1:10. It should be noted that PCR for the analysis of splicing patterns reflects the proportion of ASI(+):ASI(−) and ASII(+):ASII(−), but not the amount of each isoform.

Immunoblot analysis of sRyR expression

The whole membrane fraction of cerebellum was prepared as described by Kuwajima et al. (1992). For preparation of the crude sarcoplasmic reticulum fraction of the skeletal muscle, the tissue was homogenized with a Polytron in a solution containing 5 mM Tris/maleate (pH 7.0), 0.1 M NaCl, 2.5 mM EGTA (pH 8.0), 0.1 mM polymethanesulphonyl fluoride, 10 μM leupeptin, 10 μM pepstatin A and 1 mM 2-mercaptoethanol and was centrifuged at 4000 g (Beckman Type 70.1Ti) for 30 min. The supernatant was filtered through two layers of cheesecloth and centrifuged at 100000 g (Beckman Type 70.1Ti) for 30 min to precipitate the crude sarcoplasmic reticulum. Immunoblot detection of sRyR in the cerebellar whole membrane and the crude sarcosplasmic reticulum of skeletal muscle was performed with anti-C2 antibody as described previously (Kuwajima et al., 1992). Peroxidase-coupled detection was carried out by the enzyme-linked chemiluminescence method with an ECL kit (Amersham). Anti-C2 stains both sRyR and cRyR in cerebellum. sRyR shows slower mobility than cRyR on SDS/PAGE.

RESULTS

Identification of two alternatively spliced sites in sRyR

We amplified the entire coding region of sRyR cDNA from mouse skeletal muscle by PCR using 40 pairs of primers at overlapping intervals. By sequence analysis of the PCR products, we identified two regions that showed heterogeneity. At one region, the inclusion and the exclusion of a stretch of 15 bp (ASI) generated two variants ASI(+) and ASI(−), respectively, and at another site, the inclusion and the exclusion of a stretch of 18 bp (ASII) generated two variants ASII(+) and ASII(−), respectively (Figure 1). Exclusion of ASI and ASII results in the absence of five and six amino acid residues, respectively; these correspond to Ala^{3481}–Gln^{3485} and Val^{3865}–Asn^{3870} of rabbit sRyR. The cDNA sequences flanking ASI were identical in ASI(+) and ASI(−). Also, in ASII(+) and ASII(−), the cDNA sequences flanking ASII were identical.

We next analysed the genomic organization around ASI and ASII. PCR with primers S75 and S72, which span ASI, and primers S79 and S74, which span ASII, generated single products of about 1.7 and 1.5 kbp, respectively, from the genomic DNA. Comparison of the sequences of these fragments with the cDNA sequence revealed the exon/intron organization around ASI and

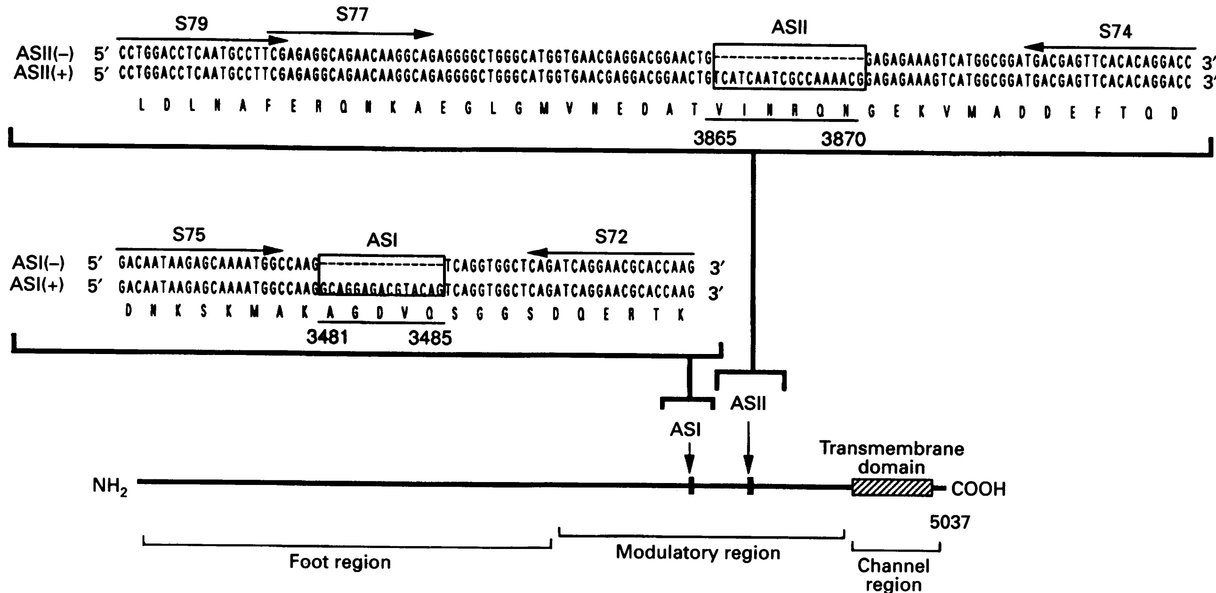

Figure 1 Schematic diagram of mouse sRyR showing the locations of two alternatively spliced regions

The cDNA sequences of ASI(−) and ASII(−) are aligned with those of ASI(+) and ASII(+) respectively. The corresponding amino acid residues are shown below the cDNA sequences in a single-letter code. Underlined sequences indicate 5 and 6 residues absent in ASI(−) and ASII(−) respectively. Amino acid residues are numbered in accordance with the sequence of rabbit sRyR. The PCR primers used in this study are indicated by arrows.

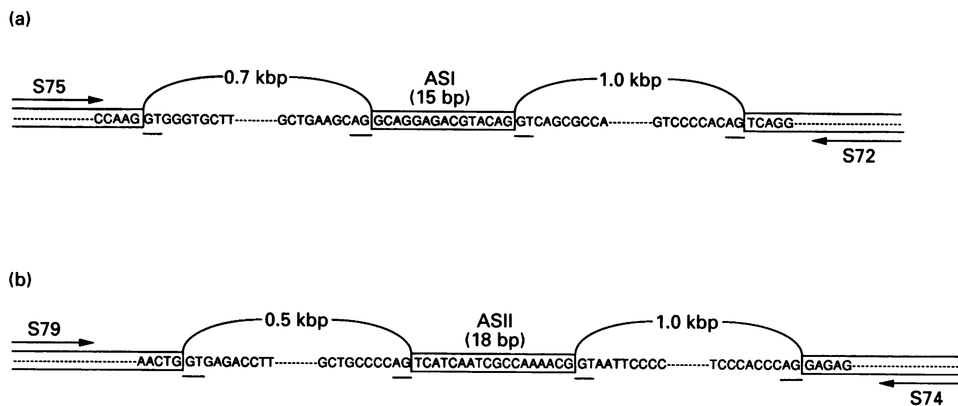

Figure 2 Arrangement of introns and exons around ASI (a) and ASII (b)

The PCR primers used to amplify the genomic DNA fragments are indicated by arrows. Exons are represented by boxes. Nucleotides at the splice sites match the GT/AG rule (underlined).

ASII (Figure 2). ASI corresponded to an exon of 15 bp flanked by introns of 0.7 and 1.0 kbp, and ASII corresponded to an exon of 18 bp flanked by introns of 0.5 and 1.0 kbp. All the sequences of exon/intron boundaries were in accordance with the GT/AG rule (Green, 1991). These results show that the heterogeneities of mRNA at ASI and ASII are generated by alternative use of these 15 bp and 18 bp exons.

Splicing patterns of ASI and ASII are tissue-specific

sRyR was expressed in brain (Kuwajima et al., 1992; Furuichi et al., 1994). We detected mRNA of sRyR also in heart although the expression level was very low (less than 2 % of skeletal muscle as probed by RNase protection assay, results not shown). We then examined whether ASI and ASII were differently spliced among skeletal muscle, cerebrum, cerebellum and heart of the adult mouse. The ratios ASI(+):ASI(−) and ASII(+):ASII(−) were estimated by PCR analysis in which mixtures of cloned cDNA fragments containing and lacking ASI or ASII were used as standard templates (see the Experimental section). PCR with primers S75 and S72 generated products of 70 and 55 bp, which corresponded to ASI(+) and ASI(−), respectively (Figure 3a). In skeletal muscle, both ASI(+) and ASI(−) were detected. The molar ratio ASI(+):ASI(−) was about 3:1. In contrast, only ASI(+) was detected in cerebellum. In cerebrum, we detected only a very small amount of ASI(+). In heart, we could detect neither ASI(+) nor ASI(−) probably because of their low level of expression and the sensitivity of the method. Indeed, we could

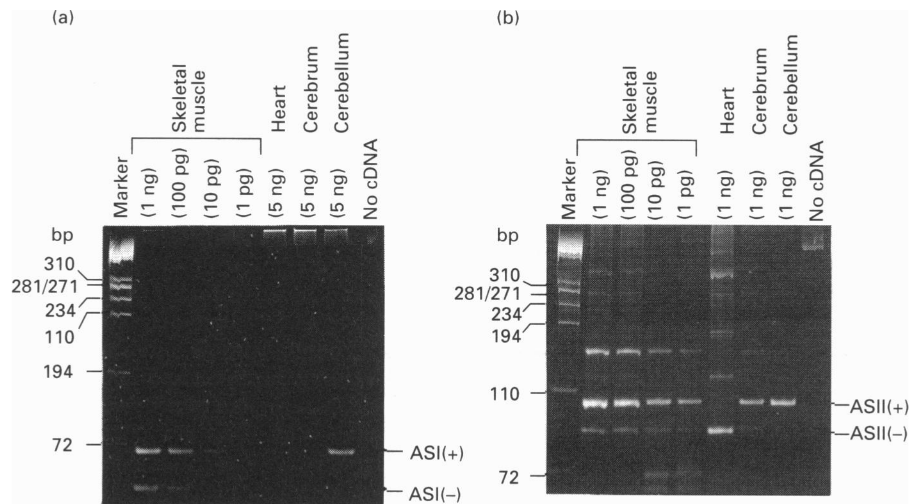

Figure 3 PCR analysis of splicing patterns at ASI and ASII in adult mouse tissues

sRyR cDNA from skeletal muscle, heart, cerebrum and cerebellum were amplified with the primers S75 and S72 (**a**), and S77 and S74 (**b**). The products were analysed on polyacrylamide gel. The amounts of cDNA templates are shown. The PCR products of 70 and 55 bp which correspond to ASI(+) and ASI(−) respectively (**a**), and those of 110 and 92 bp which correspond to ASII(+) and ASII(−) respectively (**b**) are indicated.

not detect sRyR in as little as 1 pg of skeletal muscle cDNA. PCR with primers S77 and S74 generated products of 110 and 92 bp, which corresponded to ASII(+) and ASII(−), respectively (Figure 3b). In skeletal muscle and cerebrum, both ASII(+) and ASII(−) were detected. The molar ratios ASII(+):ASII(−) were about 10:1 in skeletal muscle and more than 10:1 in cerebrum. In cerebellum, only ASII(+) was detected. In contrast, only ASII(−) was detected in heart.

Splicing patterns of ASI and ASII are developmentally regulated

We analysed the splicing pattern in mouse tissues at various stages of development by PCR using the same primers as used in the analysis of the tissue-specificity of the splicing pattern. In skeletal muscle, the splicing pattern of ASI varied dramatically (Figure 4b). Only ASI(−) was detected in the embryonic period and the proportion of ASI(+) increased gradually with development. Also, the splicing pattern of ASII changed dramatically (Figure 4c). At postnatal day 0 (P0), ASII(−) abruptly became predominant. Then the ratio ASII(+):ASII(−) increased gradually until P21. Immunoblot analysis showed that the expression of sRyR increased gradually from E14 to P21 (Figure 4a). Therefore, the change in the splicing patterns must be due to the increase in the expression levels of ASII(−) upon birth and of ASI(+) and ASII(+) after birth. In cerebrum, the splicing pattern of ASII showed a striking change during the embryonic period (Figure 5). ASII(−) was exclusively present at E14 whereas ASII(+) was predominant at embryonic day 18 (E18). Thereafter, the ratio ASII(+)/ASII(−) did not change. In heart, ASII(−) but not ASII(+) was detected throughout development (results not shown). We do not know the splicing pattern at ASI in cerebrum and heart because we could not amplify the corresponding regions. In cerebellum, the expression level of sRyR increased rapidly after P10 as shown by immunoblot analysis (Figure 6a), and the splicing patterns of ASI and ASII scarcely changed (Figure 6b and c). As for ASI, only ASI(+) was detected throughout the development. As for ASII, ASII(+) was predominant from P8 to P21; the ratio ASII(+):ASII(−) was about 3:1. In adults, only ASII(+) was detected. These results show that the splicing pattern of ASII in the cerebellum varied slightly after P21. We could not detect sRyR cDNA in cerebellum until P8, probably because of the very low level of expression.

DISCUSSION

sRyR plays a central role in the excitation–contraction coupling of skeletal muscle. sRyR is expressed also in brain (Kuwajima et al., 1992; Furuichi et al., 1994) and, as shown in the present study, in heart. These results suggest that sRyR also plays roles in other tissues besides skeletal muscle. In the present study, we identified two alternatively spliced region, ASI and ASII, in mouse sRyR mRNA. The splicing pattern of these two regions varied among tissues and during development.

The exclusion of ASI and ASII leads to the absence of the regions corresponding to Ala^{3481}–Gln^{3485} and Val^{3865}–Asn^{3870} respectively, in rabbit sRyR. These amino acid sequences are present in the modulatory region, where various modifications are known or postulated to occur. Ca^{2+} binding at 4240–4379 and 4364–4529 (Chen et al., 1992) and phosphorylation of Ser^{2843} (Suko et al., 1993) have been shown. Binding of calmodulin (at 2807–2840, 2909–2930, 3031–3049, 3614–3637 and 4295–4325) and ATP (at 4449–4454 and 4452–4457) and phosphorylation (at 3940–3945 and 4314–4317) are predicted (Takeshima et al., 1989; Zorzato et al., 1990). Indeed, channel activity of RyR in skeletal muscle is inhibited in the presence of calmodulin (Meissner, 1986). Wang and Best (1992) suggested that sRyR was inactivated by Ca^{2+}/calmodulin-dependent protein kinase II, whereas Herrmann-Frank and Varsanyi (1993) showed that phosphorylation enhanced channel activity of the purified sRyR. Furthermore, ATP stimulates Ca^{2+} release via RyR in skeletal muscle (Meissner et al., 1986). Possibly, the alternative splicings at ASI and ASII affect some of these modifications; the resultant isoforms must show heterogeneities in channel activity, response to depolarization of the plasma membrane and cytosolic Ca^{2+} concentration, interaction with other proteins, and intracellular

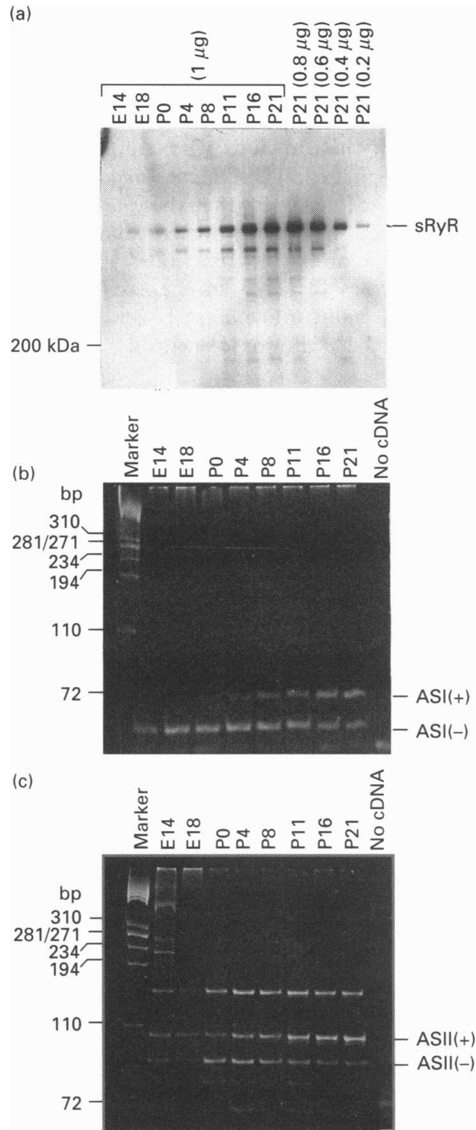

Figure 4 Expression and splicing pattern of sRyR in skeletal muscle from E14 to P21

(**a**) Immunoblot analysis of sRyR expression. The amounts of the protein of crude sarcoplasmic reticulum applied to the SDS/PAGE gel are shown. The position of sRyR is indicated. (**b**) and (**c**) PCR analysis of the splicing pattern at ASI (**b**) and ASII (**c**). cDNA was amplified with PCR primers S75 and S72 (**b**) and S77 and S74 (**c**). The products were analysed on polyacrylamide gel. PCR products corresponding to ASI(+), ASI(−), ASII(+) and ASII(−) are indicated.

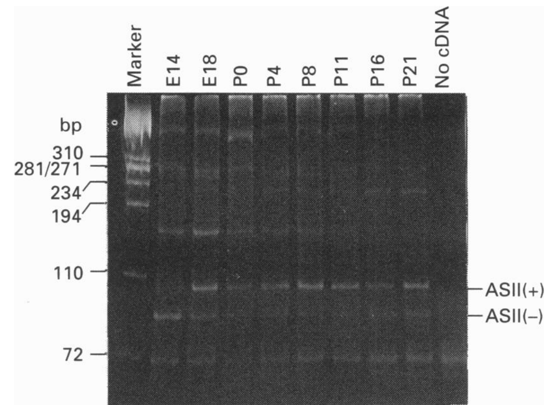

Figure 5 Splicing pattern at ASII of sRyR during mouse cerebrum development

cDNAs from E14 to P21 were amplified with PCR primers S77 and S74, followed by polyacrylamide gel electrophoresis. PCR products corresponding to ASII(+) and ASII(−) are indicated.

localization. It is interesting also that cRyR and type I IP_3R have alternatively spliced regions near the putative phosphorylation sites upstream of the transmembrane region (Nakai et al., 1990; Nakagawa et al., 1991). Alternative splicing in the modulatory region may be a common strategy to regulate the function of the Ca^{2+} release channels. In addition, near ASII there are two regions (3886–3896 and 3965–3973) highly homologous to type I IP_3R (Hakamata et al., 1992); these regions may play common roles in the two Ca^{2+} release channels. The alternative splicing at ASII may affect such roles of sRyR.

Except in the modulatory region, we could find no splicing variants. This is in contrast to the type I IP_3R in which alternative splicing is observed in the N-terminal region in addition to the modulatory region. Although the structural outlines of sRyR and type I IP_3R are similar, their N-terminal regions show little sequence homology and play different roles. The N-terminal region of IP_3Rs binds Ins(1,4,5)P_3, and alternative splicing in this region seems to generate IP_3Rs with a different affinity for Ins(1,4,5)P_3 (Nakagawa et al., 1991; Miyawaki et al., 1991). This heterogeneity must be useful for generating the diversity of the Ins(1,4,5)P_3 signalling mechanism in different types of cells. The N-terminal region of sRyR is considered to be involved in the interaction with the voltage-gated Ca^{2+} channel of the plasma membrane (Takeshima et al., 1989). This interaction must mediate depolarization-induced Ca^{2+} release in skeletal muscle and seems to be a specific function of sRyR. The absence of alternative splicing in the N-terminal region suggests that heterogeneity of such an interaction is unnecessary or even disadvantageous for skeletal muscle.

The change in splicing patterns during development is interesting. In skeletal muscle, ASII(−) increased abruptly between E18 and P0. During this period, myotubes turn into young myofibers (Platzer, 1978); the increase in ASII(−) may participate in this histological change. Postnatally, young myofibers become matured: the number of sarcoplasmic reticula increases and triad junctions align transversely at the A–I band (Platzer, 1978). sRyR is highly localized on the sarcoplasmic reticulum at the triad junction (Fleischer and Inui, 1989). Therefore the gradual increases in ASI(+) and ASII(+) after birth are probably important for the maturation of skeletal muscle. In contrast to skeletal muscle, only ASII(−) was detected from E14 to adult in heart. The sRyR, which lacks ASII, may be involved in a physiological role specific to cardiac muscle. It is interesting also that the cRyR cDNA from the heart of rabbit (Nakai et al., 1990; Otsu et al., 1990) and mouse (results not shown) lacks a region of 18 bp that corresponds to ASII. In cerebrum, a cortical layer becomes clear between E14 and E18. The drastic change in the splicing pattern of ASII between these days may be coupled with this phenomenon, which must be associated with the proliferation, maturation and migration of neurons. In contrast, the splicing pattern of ASII in cerebellum changed at the later stage, after P21. Previous studies showed that sRyRs are present exclusively in Purkinje cells (Kuwajima et al., 1992; Furuichi et al., 1994). Altman (1972) reported that in the rat,

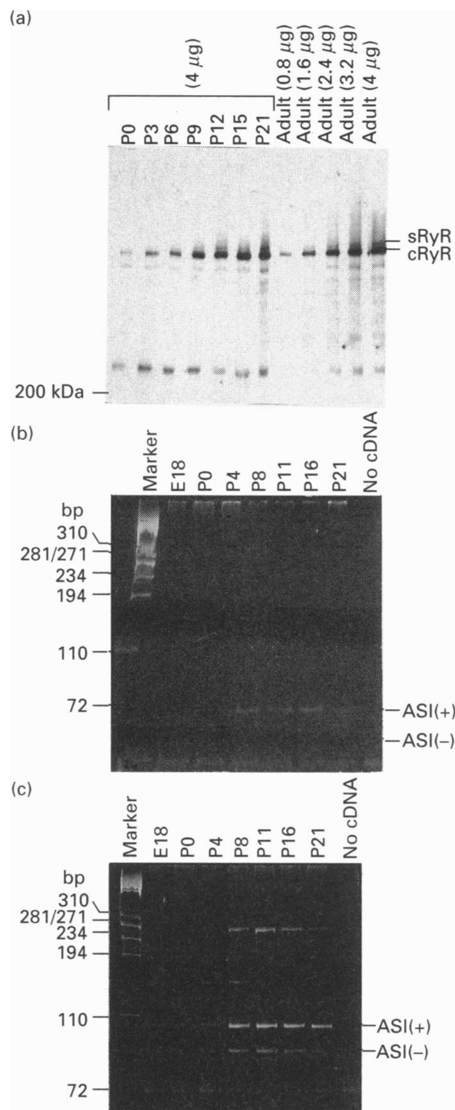

Figure 6 Expression and splicing pattern of sRyR during mouse cerebellum development

(**a**) Immunoblot analysis of sRyR expression after P0. The amounts of protein of the microsome fraction applied to the SDS/PAGE gel are shown. The positions of sRyR and cRyR are indicated. (**b**) and (**c**) PCR analysis of the splicing pattern at ASI (**b**) and ASII (**c**) from E18 to P21. cDNA was amplified with PCR primers S75 and S72 (**b**) and S77 and S74 (**c**). The products were analysed on polyacrylamide gel. PCR products corresponding to ASI(+), ASI(−), ASII(+) and ASII(−) are indicated.

rapid growth of terminal branchlets of dendrites of Purkinje cells and their formation of synapses with parallel fibres of granule cells is the most prominent event after P21. We propose that the change in the splicing pattern of ASII is involved in this synaptogenesis.

Alternative splicings at ASI and ASII seem to be conserved among mammalian systems. sRyR isoforms lacking the regions corresponding to ASI and ASII have been reported in humans (Zhang et al., 1993). In rabbits, sRyR isoform lacking the region corresponding to ASI has also been found (Zorzato et al., 1990). These reports examined the sRyR isoforms only in adult skeletal muscle. In the present study, we show for the first time that the splicing patterns of these regions vary among tissues and are developmentally regulated. These facts, together with the locations of ASI and ASII, suggest that these alternative splicings modify sRyR function. Elucidation of the effects of these alternative splicings on sRyR function will provide further insights into the roles of sRyR in the excitation–contraction coupling of skeletal and cardiac muscles, and in Ca^{2+} signalling accompanied with the excitation of neurons.

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