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A brain-specific transcript from the 3′-terminal region of the skeletal muscle ryanodine receptor gene

Hiroshi Takeshima[a,b], Seiichiro Nishimura[b], Miyuki Nishi[a], Michiko Ikeda[c] and Tetsuo Sugimoto[c]

[a]*International Institute for Advanced Studies, Shimadzu N-80, 1 Nishinokyo-Kuwahara-cho, Kyoto 604, Japan,* [b]*Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan* and [c]*Department of Anatomy, Kansai Medical University, Moriguchi, Osaka 570, Japan*

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We have shown previously that the skeletal muscle ryanodine receptor mRNA of ~16,000 nucleotides codes 5,037 amino acid residues constituting the calcium release channel in skeletal muscle. In this study, RNA blot hybridization analysis shows that the brain contains an RNA species with an estimated size of ~2,400 nucleotides hybridizable with the 3′-terminal region of the skeletal muscle ryanodine receptor cDNA. cDNA cloning and genome analysis indicated that two transcripts differing in their start sites are produced from the skeletal muscle ryanodine receptor gene in a tissue-specific fashion, and that the mRNA in brain may code the carboxyl-terminal region of the ryanodine receptor molecule. cDNA expression experiments suggested that the ATG triplet encoding Met^{4382} of the skeletal muscle ryanodine receptor can function as a translation initiation codon, and that the expressed protein composed of the carboxy terminal 656 amino acid residues of the receptor is located on the endoplasmic reticulum membrane.

Ryanodine receptor; Calcium release channel; Rabbit brain; RNA blot hybridization; cDNA expression

1. INTRODUCTION

The ryanodine receptor functions as a calcium-induced calcium release channel and takes part in Ca^{2+} release from intracellular stores [1,2]. Skeletal muscle sarcoplasmic reticulum (SR) provides the richest source of the ryanodine receptor. The purified receptor was shown to form a homo-tetrameric complex and was identified with the 'foot' structure which spans the gap between the SR and transverse tubule membranes [2]. Cloning and sequence analysis of cDNA have revealed that the rabbit skeletal muscle ryanodine receptor is composed of 5037 amino acid residues, comprising the carboxy-terminal channel region which has four putative transmembrane segments, and the remaining portion which apparently constitutes the 'foot' structure [3]. Previous observations indicated that the ryanodine receptor expressed from the cDNA is localized to calcium pools of the endoplasmic reticulum (ER) and functions as a calcium release channel in Chinese hamster ovary (CHO) cells [4,5].

Calcium-induced calcium release has been observed in a variety of cell types, including neurons [6–8]. cDNA cloning studies have previously shown that there are three types of the ryanodine receptor in mammalian tissues, the skeletal muscle, the cardiac, and the brain ryanodine receptor [3,9–12]. Our recent studies have shown that the brain contains the cardiac and the brain ryanodine receptor types, and the distribution of the receptors in rabbit brain has also been investigated by RNA blot hybridization analysis [12]. This report deals with a brain-specific RNA species derived from the 3′-region of the skeletal muscle ryanodine receptor gene.

Correspondence address: H. Takeshima, International Institute for Advanced Studies, Shimadzu N-80, 1 Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto 604, Japan. Fax: (81) (75) 811 8186.

Abbreviations: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; CHO cell, Chinese hamster ovary cell.

2. MATERIALS AND METHODS

2.1. *RNA blot hybridization analysis*

Total RNA was extracted from rabbit tissues by the guanidinium thiocyanate/cesium chloride method [13] and poly(A)$^+$ RNA was purified using oligo(dT)-cellulose [14]. RNA samples were denatured with glyoxal [15], electrophoresed on 1% agarose gels and transferred to Biodyne nylon membranes (Pall). The probes used were the *Sma*I(13290)/*Sma*I(15181) fragment from the skeletal muscle ryanodine receptor cDNA clone pRR616 [3]; restriction endonuclease sites are identified by number (in parentheses) indicating the 5′-terminal nucleotide generated by cleavage. The probes were labelled with [α-^{32}P]dCTP as in [16]. The hybridization and washing condition were the same as in [17]. Autoradiography was performed at −80°C with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) or *Hin*dIII cleavage products of λ phage DNA were used as size markers.

2.2. *DNA cloning*

Oligo(dT)-primed rabbit brain cDNA libraries [10,12] were screened with the *Sma*I(13290)/*Sma*I(15181) fragment derived from clone pRR616 [3] to yield 12 clones. The cDNA inserts of these clones were subcloned into pBluescript (Stratagene) and analysed with re-

striction endonucleases (digestion with *Rsa*I and with *Ban*I). All clones yielded fragments common to those derived from the 3′-terminal region of the skeletal muscle ryanodine receptor cDNA. Among the cDNA clones, 8 clones were further analysed as shown below. The clone λBRR2 carries nucleotides 13899 to 15230 and the poly(dA) tract (nucleotide residues are numbered as in [3]); λBRR6 (13583–15230 and the poly(dA) tract); λBRR4 (13549–15230 and the poly(dA) tract); λBRR3 (13481–15230 and the poly(dA) tract); λBRR319 (13456–15230 and the poly(dA) tract); λBRR313 (13347–15230 and the poly(dA) tract); λBRR308 (13104–15230 and the poly(dA) tract); λBRR310 (13082–15230 and the poly(dA) tract). The cDNA sequence of the λBRR2 was determined [18] and both ends of the cDNAs carried by the other 7 clones were sequenced (~ 250 nucleotides). These data showed that the brain-cDNA sequence is identical to that of the skeletal muscle ryanodine receptor cDNA corresponding to residues 13082 to 15230. Some clones (λBRR3, λBRR4 and λBRR319) include differences in the 3′-noncoding sequence as follows: GCCC for ACT at 15175–15177 and GTC for AG at 15212–15213.

A synthetic primer complementary to nucleotide residues 13315–13335, prepared using an automatic DNA synthesizer (Applied Biosystems), was elongated by the procedures described previously [3] using rabbit brain poly(A)$^+$ RNA. The resulting clones were selected with the 0.21 kbp *Eco*RI (vector)/*Sma*I (13290) fragment from λBRR310 to yield 11 positive clones including λBRR401 (12931–13335), λBRR406 (13060–13335), λBRR410 (13058–13335), λBRR413 (13031–13335) and λBRR417 (12922–13335). Sequence analysis of these clones showed that they carry partial sequences of the skeletal muscle ryanodine receptor cDNA.

A rabbit genomic DNA library [19] was screened with the 0.21-kbp *Pst*I (vector)/*Sph*I (13146) fragment from λBRR401 to yield λBRRG31. The ~ 1.8-kbp *Pst*I fragment and the ~ 3.0-kbp *Sma*I fragment from λBRRG31 were subcloned into pUC 18 or pBluescript to analyse the sequence. The genomic sequences corresponding to residues 12628 to 13434, 13435 to 13505 and 13506 to 13656 of the skeletal muscle ryanodine receptor cDNA are uninterrupted. Their sequences at the exon-intron boundaries are consistent with the GT-AG rule. The nucleotide differences observed between cDNA and genomic DNA clones are as follows: A (cDNA clone) or G (genomic) at 13446 and C (cDNA) or T (genomic) at 13487. Both of the substitutions cause no differences in amino acid sequence.

2.3. *cDNA expression in CHO cells*

The 2.1-kbp*Bst*EII (13122)/*Hin*cII (vector) fragment from pRR616 [3] was blunted and then subcloned into the *Hin*cII site of pSP 64 to yield pRRS12; a one-base-pair deletion from the *Hin*cII site ligated to the 5′-terminal *Bst*EII site of the cDNA occurred during the construction. The 2.1-kbp *Hin*dIII fragment from pRRS12 was cloned into the *Hin*dIII site of pKNH [20,21] to yield an expression plasmid pRRS13. In this plasmid the cDNA insert was positioned downstream of and in the same orientation as the SV 40 promoter. If the Met^{4382} would function as the initiation methionine, the mRNA generated from pRRS13 would code the protein composed of the carboxy terminal 656 amino acid residues of the skeletal muscle ryanodine receptor. CHO cells grown in alpha-modified MEM supplemented with 10% calf serum were transfected with *Pvu*I-cleaved pRRS13 [22]. Clones C13224 and C13238 were isolated by screening G418-resistant clones by RNA blotting analysis.

A Wistar rat was repeatedly immunized with the purified rabbit skeletal muscle ryanodine receptor [3] and polyclonal antibody was partially purified by ammonium sulfate fractionation from antiserum collected 12 days after booster injection. This antibody specifically recognized both native and denatured forms of the ryanodine receptor protein. For immunoblotting analysis, membrane preparations (100 μg protein) from CHO clones were electrophoresed on an SDS-7% polyacrylamide gel and transferred to GVHP filter (Millipore) as described [23]. The filter was incubated with the polyclonal antibody against the ryanodine receptor (~ 0.2 μg/ml) and then with [^{125}I]anti-rat immunoglobulins (New England Nuclear). After washing, the filter was subjected to autoradiography. A high molecular weight standard mixture (Sigma) was used as size marker.

For immunohistochemical analysis, the transformed cells were fixed with 2% paraformaldehyde, 1% picric acid, 0.05% glutaraldehyde and 0.15 M sodium phosphate buffer (pH 7.3) for 10–20 min at room temperature. Non-transfected CHO cells were similarly treated and served as controls for the subsequent immunohistochemical staining. Following rinsing with phosphate buffered saline (PBS), the polyclonal antibody (~ 0.3 μg/ml) was incubated with cells for 1 h at room temperature. After washing with PBS, the cell preparations were incubated with biotinylated rabbit anti-rat IgG and avidin-biotinylated peroxidase complex (Vector Labs), and then reacted with diaminobenzidine-4HCl and hydrogen peroxide. For light microscopy, the immunostained cells were washed with PBS, coverslipped with glycerine, and examined under bright-field illumination. For electron microscopy, the immunostained cells were osmicated, dehydrated, and embedded in Epon, and serial ultrathin (silver-gold) sections were prepared from Epon blocks.

3. RESULTS AND DISCUSSION

3.1. *Identification of a brain-specific 2.4-kb RNA species*

As reported previously, the rabbit brain contains the cardiac and the brain ryanodine receptor mRNA [10–12]. To investigate whether the skeletal muscle ryanodine receptor mRNA exists in brain, we performed RNA blot analysis using a set of the cDNA fragments as probes. Fig. 1A shows the result of the analysis using a probe derived from the 3′-terminal region of the skeletal muscle ryanodine receptor cDNA. Skeletal muscle contains the skeletal muscle ryanodine receptor mRNA with an estimated size of ~ 16-kb as described previously [3]. No hybridizable RNA species with a similar size could be detected in other tissues examined [12]. However, the brain contains a hybridizable RNA species with an estimated size of 2.4 kb, which could be enriched by oligo-dT affinity chromatography. This RNA species in the brain could not hybridize with probes derived from the 5′-terminal or middle portion of the skeletal muscle ryanodine receptor cDNA. Probes from the cardiac and the brain ryanodine receptor cDNA did not detect this RNA species [12]. These results indicate that the brain contains a poly-adenylated 2.4-kb RNA which possesses a nucleotide sequence identical or highly homologous with that of 3′-terminal region of the skeletal muscle ryanodine receptor mRNA. As shown in Fig. 1B, the contents of this RNA species do not differ dramatically between fetal and adult brain.

3.2. *Characterization of the 2.4-kb RNA in brain*

To further characterize the 2.4-kb RNA, we screened cDNA libraries derived from rabbit brain poly(A)$^+$ RNA using a probe from the skeletal muscle ryanodine receptor cDNA, and obtained several clones. Restriction endonuclease analysis showed that all these clones yielded fragments common to those derived from the 3′-terminal region of the skeletal muscle ryanodine receptor cDNA. Sequence analysis showed that these

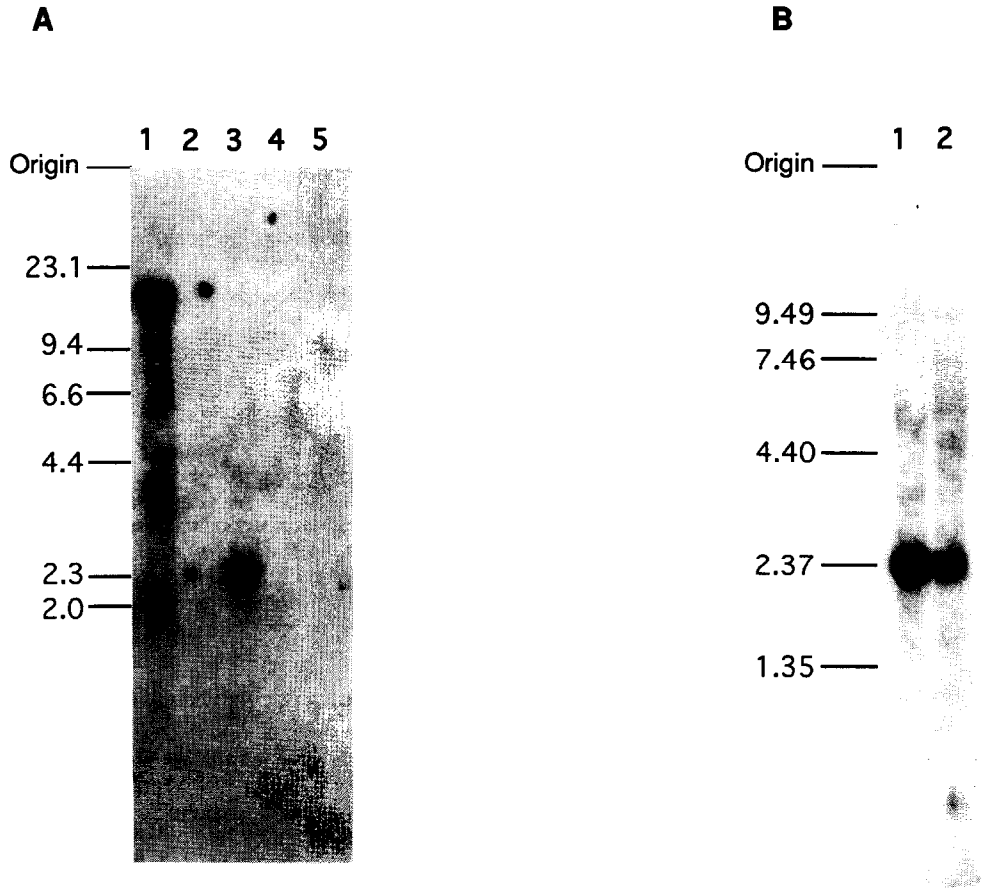

Fig. 1. Autoradiogram of blot hybridization analysis of RNA from rabbit tissues with a cDNA probe for the 3′-terminal region of the skeletal muscle ryanodine receptor mRNA. (A) Total RNA from skeletal muscle (3 μg, lane 1) and 10 μg of poly(A)$^+$ RNA from heart (lane 2), brain (lane 3), stomach (lane 4) and kidney (lane 5) were analysed as in section 2.1. All the tissues were derived from an adult rabbit. (B) 10 μg of poly(A)$^+$ RNA from adult (lane 1) and fetal (3 to 5 days before birth) brain (lane 2) were analysed. The size markers (in kb) are indicated.

clones carry parts of the sequence of the skeletal muscle ryanodine receptor cDNA. We also cloned rabbit genomic DNA corresponding to the 3′-terminal region of the skeletal muscle ryanodine receptor cDNA, and determined four exon–intron boundaries in the gene. These DNA cloning results are summarized in Fig. 2. The cDNA sequence with 2309 nucleotides (excluding the poly(dA) tract) was obtained from brain cDNA libraries and was shown to be identical to the partial sequence of the skeletal muscle ryanodine receptor cDNA, corresponding to the residues 12922 to 15230 (see section 2.2.). Residue 12922 is assumed to be close to the start site of the brain mRNA, because (i) the predicted mRNA length of 2309 plus ~ 100 to ~ 150 (the poly (A) tail) nucleotides agrees well with the observed length of 2.4-kb, and (ii) our recent cloning strategy using the primer extention method [3,11] can yield sequences close to the mRNA-cap sites. The genomic sequence corresponding to residues 12628 to 13434 of the cDNA is uninterrupted and represents an exon in the gene. The transcription start site of the 2.4-kb mRNA should be located in this exon, as otherwise a discrepancy in the size of the mRNA would occur. Interestingly, this exon contains several copies of the sequence GCGGCGGGC, and the sequence of more than 250 nucleotides around residue 13000 is highly GC-rich, as shown in Fig. 2. It is an attractive hypothesis that these motif sequences and the GC-rich region might represent brain-specific promoter and enhancer elements, respectively. It might be possible that there are several transcription initiation sites corresponding to each of the motif sequences. These DNA cloning results suggest that the brain 2.4-kb mRNA is capable of coding a protein identical with the carboxy-terminal fragment of the ryanodine receptor molecule, and that the codon for Met^{4382} is the first ATG triplet in this mRNA.

A recent study showed that the brain contains a small amount of the skeletal muscle type-ryanodine receptor with M_r ~ 400 kDa [24]. This observation does not contradict our results since the brain might contain a tiny amount of the 16-kb mRNA and its protein product derived from the skeletal muscle receptor gene.

3.3. *Expression of the 3′-terminal portion of the skeletal muscle ryanodine receptor cDNA*

To test whether the ATG triplet encoding Met^{4382} of

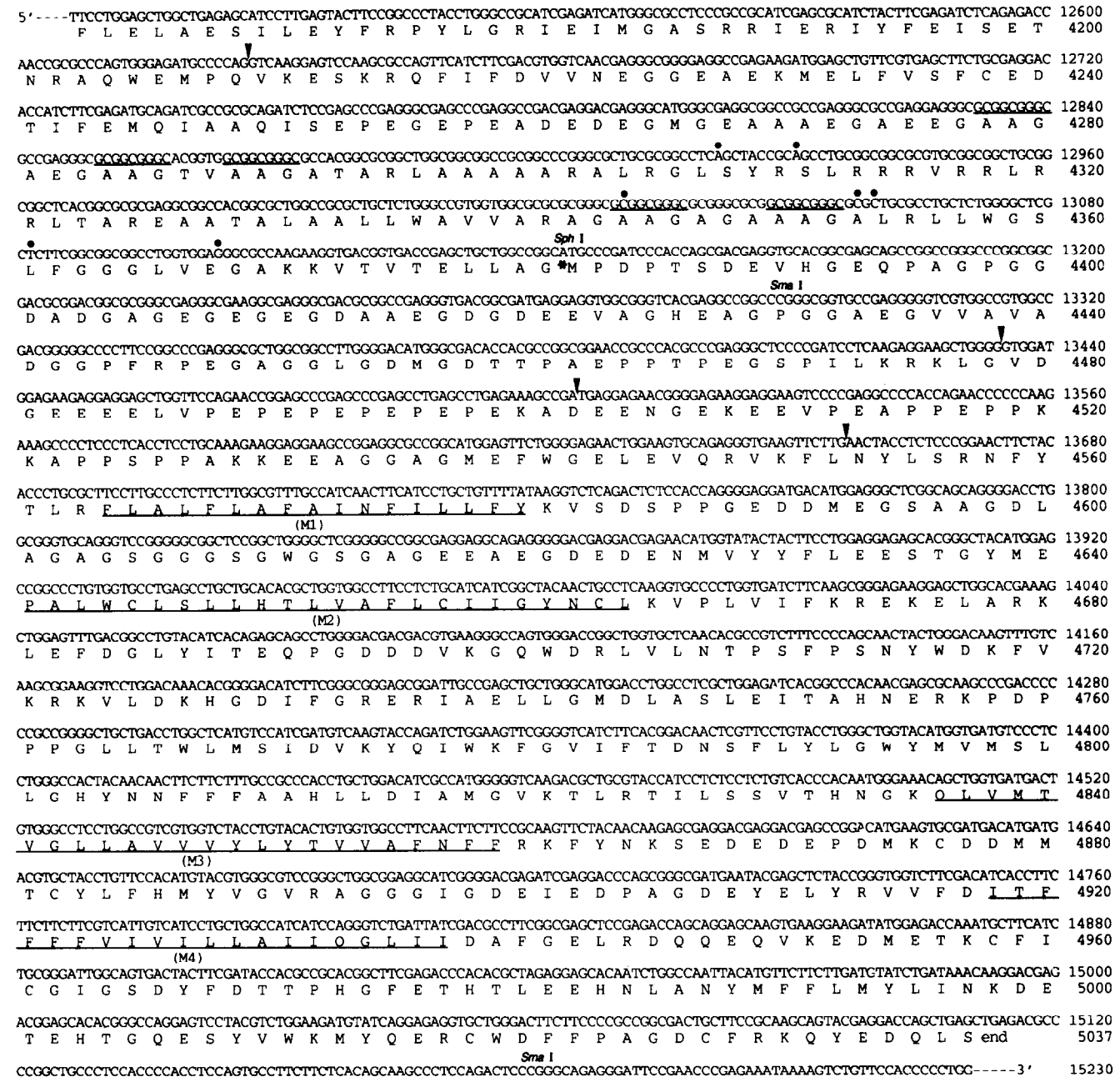

Fig. 2. Nucleotide sequence of 3'-terminal region of the rabbit skeletal muscle ryanodine receptor cDNA and the deduced amino acid sequence. The sequence data have been taken from [3]. Numbers of nucleotide and amino acid residues are given at the right of each individual line. Nucleotide residue 15230 is followed by a poly(dA) tract. The 5'-terminal residue of the sequence carried by each cDNA clone from brain libraries is indicated by filled circle (see section 2.2); the marks for the cDNA clones λBRR2, 3, 4, 6, 313 and 319 are omitted. The boundaries where the cDNA sequence is interrupted by introns in genomic DNA are shown by arrows; four exon–intron boundaries were determined in this study. Met^{4382} is marked with an asterisk and sequences of the GCGGCGGGC motif are underlined. Restriction endonuclease sites used in this study are given. The putative transmembrane segments (M1–M4) of the ryanodine receptor are also indicated.

the skeletal muscle ryanodine receptor functions as a translation initiation codon, CHO cells were transfected with an expression plasmid, pRRS13. This plasmid carries the cDNA encoding the carboxy-terminal 656 amino acid residues of the skeletal muscle ryanodine receptor (if the protein translation would start from Met^{4382}) and the neomycin-resistance marker gene. After transfection, G418-resistant clones were selected and then clones C13224 and C13238, expressing RNA species with the size expected from the expression construct, were obtained. Immunoblot analysis using polyclonal antibody to the skeletal muscle ryanodine receptor indicated that the transformed clones produced a membrane-associated protein with a relative molecular

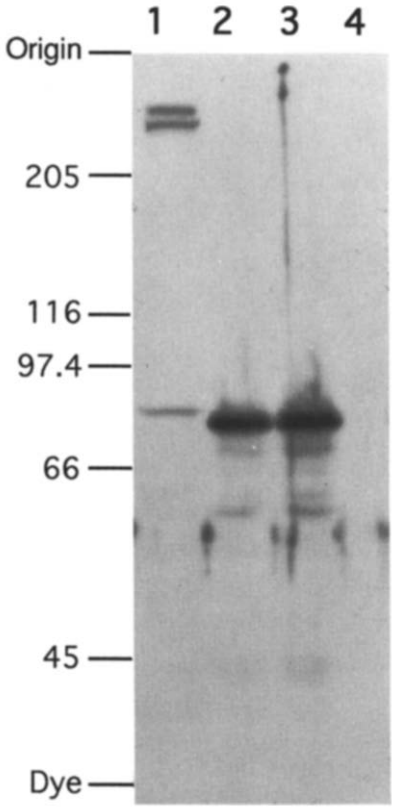

Fig. 3. Immunoblot analysis of the expressed protein in the pRRS13 transfected CHO clones. Membrane preparations (100 μg protein) from C7311 cells (lane 1), C13224 cells (lane 2), C13238 cells (lane 3) and non-transfected CHO cells (lane 4) were analysed using a polyclonal antibody against the skeletal muscle ryanodine receptor. The clone C7311 expressing the skeletal muscle ryanodine receptor was obtained previously [3]. In lane 1 the largest band (~ 400 kDa) shows the intact ryanodine receptor and other bands (~ 300 and ~ 80 kDa) are thought to be cleaved products from the intact receptor. The size markers (in kDa) are given.

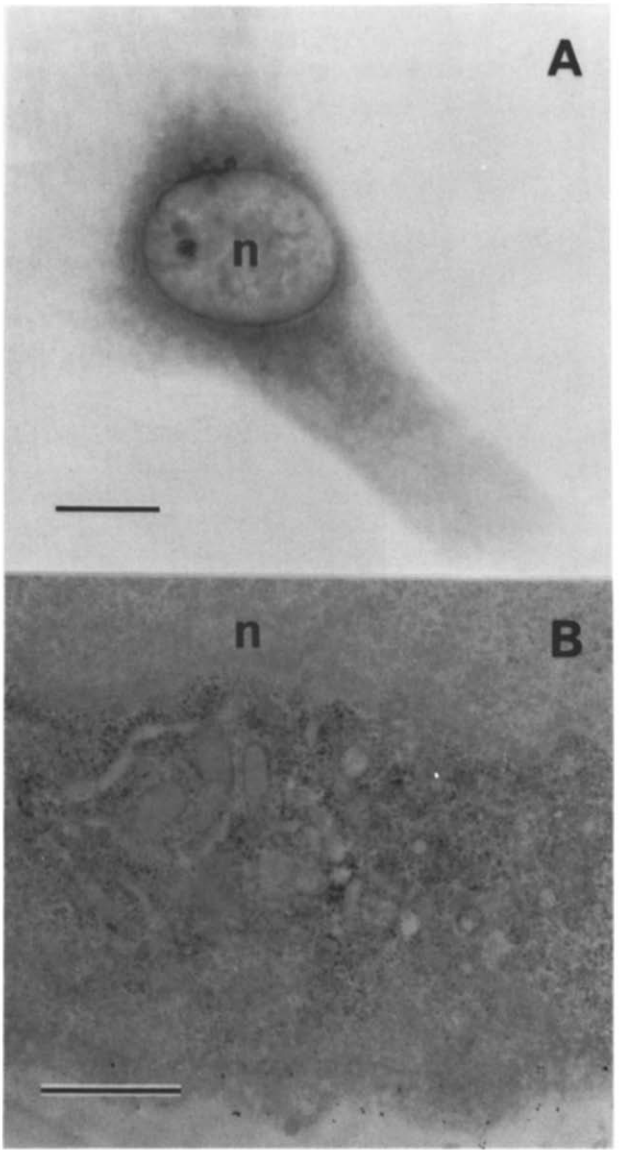

Fig. 4. Staining of pRRS13-transformed CHO cells with antibody against the skeletal muscle ryanodine receptor. (A) Photomicrograph of immunoperoxidase staining of C13238 cell, showing the expressed protein as black precipitates in the perikaryal space near the nucleus (n). Essentially the same results were also obtained in C13224 cells. Non-transfected CHO cells did not show any staining. (B) Immunoelectron microscopic profile of C13238 cell, showing the expressed protein as electron-dense particles in the perikaryal space. Note that the surfaces of endoplasmic reticulum and also those of cytoplasmic vacuoles are frequently outlined by the particles. Scale bars: 20 μm (A), 1 μm (B).

mass (M_r) of 75 kDa as in Fig. 3, in reasonable agreement with the M_r of 73.0 kDa calculated from the amino acid sequence under the assumption that the translation starts from Met^{4382}. This result indicates that the ATG triplet for Met^{4382} functions as the initiating methionine codon in CHO cells. The nucleotide sequence surrounding this ATG triplet agrees reasonably well with the consensus sequence for initiation codons [25].

Next we analysed the subcellular localization of the expressed protein using the immunoperoxidase method. As shown in Fig. 4A, positive immunostaining was obtained in pRRS13-transformed cells. Light microscopic observation of the cells revealed immunopositive precipitates in their perikaryal space adjacent to the nuclear membrane, whereas such immunolabel was far less visible in the peripheral portions or virtually absent at the cell surface. The immunoelectron micrograph in Fig. 4B shows that the perikarya of the transformed cell contain highly electron-dense particles representing the immunopositive material. Among cytoplasmic organelles, the ER was most commonly delineated by these particles. Vacuoles of various size were also outlined by these particles. Thus the surfaces of the ER are among the sites of expression of the protein. These observations suggest that ER membrane sorting signals of the ryanodine receptor molecule are in its carboxyl-terminal region. However, measurable [^{3}H]ryanodine binding or Ca^{2+} release in response to caffeine could not be de-

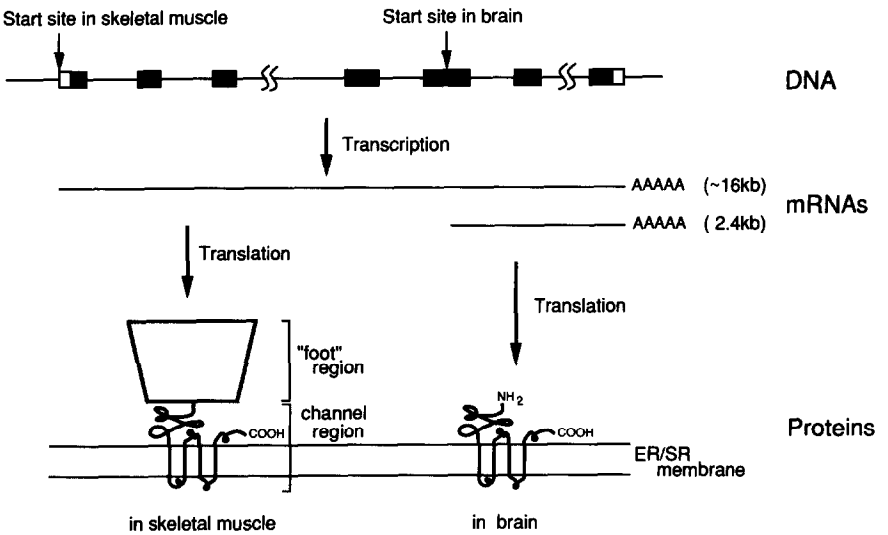

Fig. 5. Proposed scheme for the expression of the skeletal muscle ryanodine receptor gene. Molecular architecture of the ryanodine receptor is shown schematically according to our previous model [3].

tected in these transformed clones (unpublished observation), in contrast to the CHO clones transfected with the full length skeletal muscle ryanodine receptor cDNA [3,4].

According to our molecular model of the ryanodine receptor [3], this expressed protein has the channel region containing four putative transmembrane segments (M1–M4), but lacks the 'foot' region. Furthermore this protein is thought to hold the region around amino acid residue 4500 which is suggested to be involved in Ca^{2+} binding and calcium-induced calcium release in the skeletal muscle ryanodine receptor [26,27]. A possible scheme for the expression of the skeletal muscle ryanodine receptor gene is shown in Fig. 5. The possibility might arise that this protein forms homo- or hetero-tetramers contributing a variety of calcium release channels in the brain.

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REFERENCES

[1] Endo, M. (1977) Physiol. Rev. 57, 71–108.
[2] Fleischer, S. and Inui, M. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 333–364.
[3] Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Nature 339, 439–445.
[4] Penner, R., Neher, E., Takeshima, H., Nishimura, S. and Numa, S. (1989) FEBS Lett. 259, 217–221.
[5] Imagawa, T., Nakai, J., Takeshima, H., Nakasaki, Y. and Shigekawa, M. (1992) J. Biochem. (Tokyo) 112, 508–513.
[6] Kuba, K. (1980) J. Physiol. 298, 251–269.
[7] Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) Neuron 1, 355–365.
[8] Thayer, S.A., Perney, T.M. and Miller, R.J. (1988) J. Neurosci. 8, 4089–4097.
[9] Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 2244–2256.
[10] Otsu, K., Willard, H.F., Khanna, V.K., Zorzato, F., Green, N.M. and MacLennan, D.H. (1990) J. Biol. Chem. 256, 13472–13483.
[11] Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H. and Numa, S. (1990) FEBS Lett. 271, 169–177.
[12] Hakamata, Y., Nakai, J., Takeshima, H. and Imoto, K. (1992) FEBS Lett. 312, 229–235.
[13] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[14] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[15] McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4835–4836.
[16] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[17] Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) Nature 321, 406–411.
[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[19] Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978) Cell 15, 687–701.
[20] Nukada, T., Mishina, M. and Numa, S. (1987) FEBS Lett. 211, 5–9.
[21] Fukuda, K., Higashida, H., Kubo, T., Maeda, A., Akiba, I., Bujo, H., Mishina, M. and Numa, S. (1988) Nature 335, 355–358.
[22] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[24] Kuwajima, G., Futatugi, A., Niinobe, M., Nakanishi, S. and Mikoshiba, K. (1992) Neuron 9, 1133–1142.
[25] Kozack, M. (1984) Nucleic Acids Res. 12, 857–872.
[26] Chen, S.R.W., Zhang, L. and MacLennan, D.H. (1992) J. Biol. Chem. 267, 23318–23326.
[27] Fill, M., Majia-Avarez, R., Zorzato, F., Volpe, P. and Stefani, E. (1991) Biochem. J. 273, 449–457.