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Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum

(excitation–contraction coupling/calcium-release channel/BC_3H1 cells)

ANDREW R. MARKS*†, PAUL TEMPST‡§, KWANG S. HWANG*, MARK B. TAUBMAN*†, MAKOTO INUI¶, CHRISTOPHER CHADWICK¶, SIDNEY FLEISCHER¶, AND BERNARDO NADAL-GINARD*‡

*Laboratory of Molecular and Cellular Cardiology, ‡Howard Hughes Medical Institute, Department of Cardiology, Childrens Hospital, †Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, §Department of Genetics, Harvard Medical School, Boston, MA 02115; and ¶Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235

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ABSTRACT **Major progress has been made in elucidating the calcium release mechanism involved in excitation–contraction coupling. The ryanodine receptor of sarcoplasmic reticulum has been isolated and found to be morphologically identical to the foot structure, which is involved in the junctional association of terminal cisternae with the transverse tubule. The foot structure also contains the calcium release channel itself. For this reason, we refer to the foot structure as the junctional channel complex (JCC). The JCC consists of an oligomer of a single high molecular weight protein. Although progress has been made in characterizing important aspects of the structure and function of the JCC, further understanding of the JCC protein subunit awaits the molecular cloning of the JCC. We report on the isolation of cDNA clones encoding portions of the JCC from rabbit fast-twitch skeletal muscle and its tissue distribution and expression. The large size and lack of solubility of the JCC protein posed particular challenges to cloning this molecule. Among these was the necessity to develop techniques for partially digesting the JCC protein subunit with endoproteases in the presence of detergent. With this approach we obtained partial amino acid sequences from regions of the JCC and designed oligonucleotide primers and probes to synthesize and screen cDNA libraries. The rabbit skeletal muscle JCC mRNA encodes an ≈16-kilobase mRNA present in skeletal, heart, and aortic smooth muscle, as determined by RNA blot analysis with a 700-base-pair cDNA probe. Whereas the JCC mRNA appears to be relatively abundant in adult rabbit fast-twitch skeletal muscle, it is much less abundant in heart and smooth muscle. The JCC mRNA in BC_3H1 (a myoblast cell line) is reversibly regulated by growth factors in a manner similar to muscle-specific contractile protein genes.**

Muscle contraction requires elevation of calcium in the myoplasm. Uptake of calcium by the sarcoplasmic reticulum (SR) enables muscle to relax. Calcium uptake, mediated by the calcium pump protein, also referred to as the calcium ATPase, has been well characterized (1, 2). The calcium release machinery of the SR, which triggers muscle contraction, has been described in molecular terms (3).

In fast-twitch skeletal muscle, excitation–contraction coupling is thought to involve depolarization of the transverse tubule (T-tubule) resulting in rapid calcium release from the terminal cisternae of SR through the calcium release channel (CRC) (3). Coupling between the CRC and a signal transducer in the T-tubule membrane, possibly the dihydropyridine receptor has been postulated, but further studies are required to prove or disprove this hypothesis (4, 5). The structure of the α_1 subunit of the dihydropyridine receptor and its requirement for excitation–contraction coupling has been elaborated by Tanabe *et al.* (6).

Ryanodine, in nanomolar concentrations, acts as a specific ligand on the CRC, which has been localized to the terminal cisternae of SR (7). This observation led to the isolation of the ryanodine receptor and its morphological identification as the foot structure (7, 8). The feet are involved in the junctional association of T-tubules with terminal cisternae of SR to form the triad junction (9). The CRC consists of an oligomer of a single polypeptide, estimated by SDS/PAGE to have a molecular weight of 360–450,000 (8, 10, 11). The identity of the ryanodine receptor with the CRC was established by reconstitution into planar bilayers (11–13). The CRC exhibits four-fold symmetry and appears to consist of a tetramer (11, 14, 15), whose molecular weight has been estimated by scanning transmission electron microscopy to be 2.3 ± 0.3 million, equivalent to a monomer molecular weight of 570,000 (16). The three-dimensional architecture of the CRC/foot structure of SR has been obtained and the pathway of calcium release from terminal cisternae into the junctional gap is suggested from the structure (15). The foot structure is the largest channel described thus far. We proposed the term junctional channel complex (JCC) for the CRC/foot structure since it serves both as the junctional connection with the T-tubule and contains the calcium channel itself (15).

Although significant progress has been made in characterizing the JCC, cDNA clones of its mRNA can provide insight into tissue distribution and expression as well as the primary sequence of the subunit of the JCC and will lead eventually to the establishment of structure–function correlations. We now report on the isolation of cDNA clones encoding portions of the JCC. The DNA probes thereby obtained have been used to characterize the mRNA of the channel in various muscles and as a function of myogenic development.

MATERIALS AND METHODS

Enzymatic Digests of Purified JCC and Isolation of Peptides. The JCC was purified from rabbit fast-twitch skeletal muscle as described (8, 17, 18). Two 120-μl aliquots from a solution (1 mg/ml) of purified protein in a buffer containing 20 mM Tris·HCl (pH 7.4), 0.5 M KCl, 2 mM dithiothreitol, 0.5 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were digested with either 7.5 μg of *Achromobacter* endoprotease Lys-C (Wako Chemicals, Dal-

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Abbreviations: CRC, calcium release channel; JCC, junctional channel complex; SR, sarcoplasmic reticulum; T-tubule, transverse tubule; FCS, fetal calf serum.

las, TX) for 6 hr at 37°C or 3 μg of sequencing-grade *Staphylococcus aureus* V8 endoprotease Glu-C (Boehringer, Indianapolis) for 4 hr at 37°C, respectively.

SDS/PAGE. Analytical digests and small aliquots of micropreparative digests were analyzed on 8–25% gradient polyacrylamide gels containing SDS by using a PHAST system (Pharmacia, Piscataway, NJ). Gels were manually stained with silver (19).

Reverse-Phase HPLC of Peptides. Peptide mixtures resulting from each of the digests were immediately applied to a Vydac 4.6 × 250 mm C_4 reverse-phase column (214TP54, The Separations Group) in a model 150A HPLC system (Applied Biosystems) at a flow of 1 ml/min and ambient temperature. Solvent A was 0.1% trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in 70% (vol/vol) acetonitrile. Gradient I was a 10-min isocratic gradient at 25% B, 25–70% B in 45 min, and 70–100% B in 15 min; gradient II was 5–50% B in 45 min, 50–70% B in 10 min, and 70–100% B in 8 min. Peaks were collected manually and, depending on their elution position, rechromatographed on either a Vydac C_{18} or Vydac diphenyl (4.6 × 250 mm) column, as described (20).

Peptide Sequencing. Purified peptides were sequenced using an Applied Biosystems model 477A automated sequencer, optimized for subpicomole phenylthiohydantoin amino acid derivative analysis (21).

Oligonucleotides. Two antisense oligonucleotides, ACATTCAGGGTYTC (where Y is C or T), were designed based on the amino acid sequence of the C-terminal portion (Glu-Thr-Leu-Asn-Val) of one peptide KC3 (see Table 1) and pooled, designated KC3-AP (AP indicates antisense primer). KC3-AP was used as a primer to synthesize a specific primed cDNA library. A second mixture of 16 oligonucleotides KC3-PR (PR indicates probe) AARAARGCCACWGTKGATGA (where R is G or A, W is A or T, and K is G or T), based on the N-terminal portion of the amino acid sequence of peptide KC3 (Lys-Lys-Ala-Thr-Val-Asp-Ala) was used as a probe to screen the specific primed cDNA library. The synthetic oligonucleotides were synthesized on a Biosearch model 8600 DNA synthesizer.

Construction of cDNA Libraries. Total RNA (35 μg), purified from adult female New Zealand White rabbit fast-twitch skeletal muscle using the guanidine thiocyanate/cesium chloride method (22), and the KC3-AP oligonucleotide primer (2 μg) were used to construct cDNA libraries with the Pharmacia cDNA synthesis kit by the manufacturer's protocol. Double-stranded cDNA was ligated to predigested phosphatase-treated λZAPII arms (Stratagene). Aliquots of this cDNA were packaged using Gigapack Gold (Stratagene) by the manufacturer's specifications. A random-primed adult rabbit skeletal muscle library was also constructed using a similar protocol.

DNA Sequencing. Sequencing of the cDNA was carried out on both strands using the didexoynucleotide DNA sequencing techniques or an automated DNA sequencer model 370A (Applied Biosystems). cDNA sequences were analyzed using a Digital Vax computer, the University of Wisconsin Genetics Computer Group software, and the GenBank database (compared to all sequences in Genbank as of May 1989).

Northern Blot Analysis. Total RNA was prepared from adult female New Zealand White rabbits or C2, L_6E_9, or BC_3H1 cells using the guanidine/cesium chloride method (22). RNA was size-fractionated on formaldehyde/agarose gels and blotted to nitrocellulose. (RNA quantity was normalized by ethidium bromide staining of the 28S rRNA and in the BC_3H1 experiments with hybridization of an α-tubulin cDNA probe.) A cDNA probe [a 700-base-pair (bp) fragment corresponding to nucleotides 180–880 of the JCC sequence, see Fig. 2] was labeled using [α-^{32}P]dCTP (Amersham), specific oligonucleotide primers, and the Klenow fragment of DNA polymerase I (Promega) to a specific activity of 5×10^8 cpm/μg, and blots were hybridized in a buffer containing 1× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02%

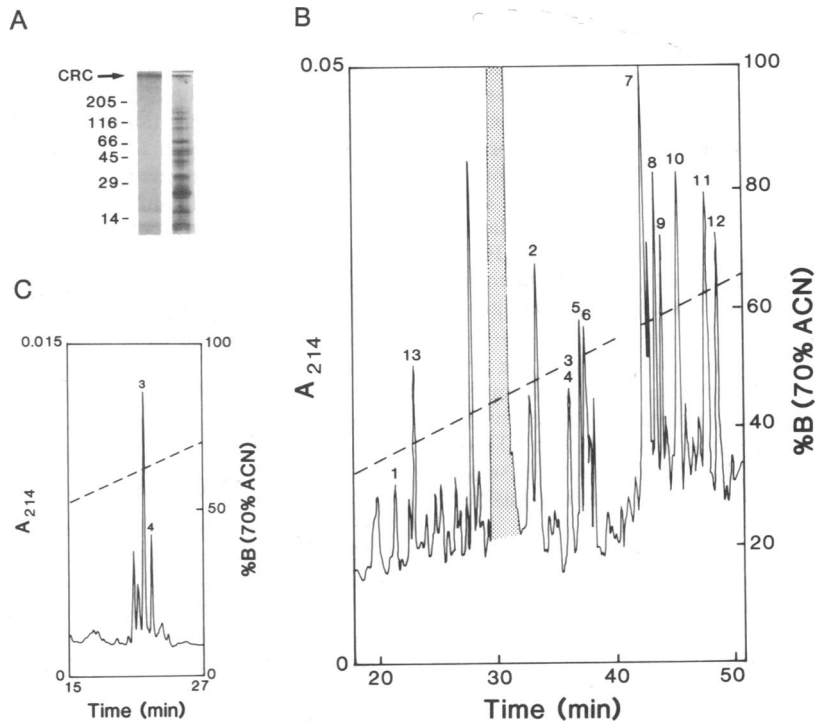

FIG. 1. Purification of peptides produced by limited digest of the JCC protein with endoprotease Lys-C. (*A*) SDS/PAGE analysis of intact protein (CRC) (lane 1) and 1% of the micropreparative digest (lane 2). (*B*) Reverse-phase HPLC separation of the digest mixture on a C_4 column by using gradient I. Numbered peaks correspond to sequenced peptides listed in Table 1. Only the relevant section of the chromatogram is shown; the shaded peak is CHAPS-related. (*C*) Repurification of a peptide fraction, labeled as "3/4" on *B*, by reverse-phase HPLC using a diphenyl column. The gradient is shown by a dashed line.

Table 1. Amino acid sequences of proteolytic peptides from the JCC protein

Peptide	Purification	Initial yield, pmol	Sequence
V1	C_4	15	NLRRSAGG(W)GEAE(GG)
V2	C_4	17	NLRRSAGGWGEA
V3	C_4	(1/5)11	LQAMAEQLAE
V4	C_4/DF	10	. . KTRKISQTAQTYDP . EGY
V5	C_4/DF	20	. LKAMIA
V6	C_4/DF	16	KWAFDKIQNN . SYGENVDE
V7	C_4/DF	12	LLAGMPDPT
V8	C_4	18	LGKQ . N . M
V9	C_4	17	VQMLMPVS . . . MPN . FLQ . E
V10	C_4	2	. LKAMIA
V11	C_4	37	FDGLYIT . QPG . D . (VK)GQ
V12	C_4	12	. F(E)PFAINMQRPVT . . F(S)K
KC1	C_4	14	EIYRWPIK
KC2	C_4/DF	5	(K)VTVTELLAGMPDPT . (D)
KC3	C_4/DF	10	KATVDAEGNFDP . PVETLNV
KC4	C_4/DF	7	(S)KL . EDYLYMAY . DIMA
KC5	C_4/DF	27	. (S)MEEQ . . . Q . EEA
KC6	C_4/DF	5	ELAR . LEF
KC7	C_4	12	. VQVETISPGDGRTF
KC8	C_4	10	TRKISQTAQTYDP . EGYN
KC9	C_4	(1/5)5	ISQTAQTYDP . E . . N
KC10	C_4	6	SLPQFEPVPPE
KC11	C_4	8	KATVDAEGNFDP
KC(SDS)2	C_4/C_{18}	35	THPMLRPY
KC(SDS)5	C_4/DF	25	EIYRWPIK

JCC peptide sequences and yields. Sequences are in the single-letter amino acid code. Residues in parentheses are tentative; dots indicate that no identification could be made. Peptide denominations are as follows: V, peptides obtained after *S. aureus* V8 Glu-C endoprotease digests; KC, peptides from a digest with *Achromobacter* Lys-C endoprotease; KC(SDS), as with KC but in the presence of 0.2% SDS. The second column lists how peptides were purified. C_4, Vydac C_4 column; DF, Vydac diphenyl column; C_{18}, Vydac C_{18} column. Initial yields of the sequencing experiments are listed; only 20% of peptides V3 and KC9 was analyzed.

Ficoll/0.02% bovine serum albumin), 5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 0.025 M sodium phosphate (pH 7.4), calf thymus DNA (50 mg/ml), 0.1% SDS, and 50% (vol/vol) formamide at 42°C. Washing was with 0.2× SSC/0.1% SDS at 42°C.

Cell Culture. BC_3H1 cultures (a generous gift of Eric N. Olson, University of Texas) were grown in 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 20% (vol/vol) fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml), as described (23). The medium was changed every 2 days. To minimize differentiation during routine passaging, BC_3H1 cells were passaged when no more than 75% confluent. To initiate differentiation, medium containing 20% FCS (growth medium) was removed and replaced with DMEM containing 0.5% FCS (low-serum medium).

RESULTS

Isolation and Sequencing of Peptides. The JCC protein was subjected to limited enzymatic proteolysis. Resulting peptides were purified by one or two rounds of reverse-phase HPLC as shown in Fig. 1 for the Lys-C digest. A number of these peptides were successfully sequenced; the results are summarized in Table 1.

Isolation of cDNA Clones. A cDNA library was constructed using KC3-AP as a primer. Two cDNA clones were isolated initially using KC3-PR as a probe. These two cDNAs (800 bp, total minus the overlap) encoded four of the peptides whose

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1    K M P L K L L T N H Y E R C W K Y Y C L

21   P T G W A N F G V T S E E E L H L T R K

41   L F W G I F D S L A H K K Y D Q E L Y R

61   M A M P C L C A I A G A L P P D Y V D A

81   S Y S S K A E K K A T V D A E G N F D P
                KC3-AP                KC3
101  R P V E T L N V I I P E K L D S F I N K

121  F A E Y T H E K W A F D K I Q N N W S Y
                                      V6
141  G E N V D E E L K T H P M L R P Y K T F
                          KC(SDS)2
161  S E K D K E I Y R W P I K E S L K A M I
                    KC(SDS)5          V10
181  A W E W T I E K A R E G E E E R T E K K

201  K T R K I S Q T A Q T Y D P R E G Y N P
                          KC8
221  Q P P D L S G V T L S R E L Q A M A E Q
                                    V3
241  L A E N Y H N T W G R K K K Q E L E A K

261  G G G T H P L L V P Y D T L T A K E K A
                     KC13A
281  R D R E K A Q E L L K F L Q M N G Y A V
                                KC13B
301  T R G L K D M E L D T S S I E K R F A F

321  G F L Q Q L L R W M D I S Q E F I A H L

341  E A V V S S G R V E K S P H E Q E I K F

361  F A K I L L P L I N Q Y F T N H C L Y F

381  L S T P A K V L G S G G H A S N K E K E

401  M I T S L F C K L A A L V R H R V S L F
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FIG. 2. Deduced amino acid sequence for a portion of the ryanodine receptor/JCC cDNA encoding 9 of the 24 JCC peptides (see Table 1). Encoded peptides are underlined. Single-letter amino acid code is used. KC3-AP denotes the sequence corresponding to the oligonucleotide used to prime the synthesis of the JCC-specific cDNA library.

sequence had been determined (Table 1 and Fig. 2). Subsequent cDNAs isolated encoded five additional peptides whose sequences had been determined (Table 1 and Fig. 2). This established the identity of these clones as coding for the JCC mRNA.

Tissue Distribution of JCC mRNA. To characterize the tissue distribution of the JCC mRNA and confirm the identity of the cDNA clones, Northern blot analysis of total rabbit RNA was performed (Fig. 3). This showed that the JCC is encoded by an ≈16-kilobase (kb) mRNA in rabbit skeletal muscle. The skeletal muscle JCC cDNA probes also hybridize to a ≈16-kb mRNA in heart and aortic muscle; however, the signal on Northern blots was considerably less. No hybridization was detected in liver and kidney RNA (Fig. 3).

Expression of the JCC Gene in BC_3H1 Cells. mRNA levels for a number of the proteins involved in contraction have

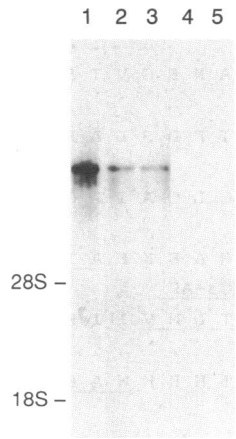

FIG. 3. Northern blot of rabbit total RNA from rabbit fast-twitch skeletal muscle (10 μg, lane 1), heart (40 μg, lane 2), aorta (40 μg, lane 3), liver (40 μg, lane 4), and kidney (40 μg, lane 5). The blot was probed with a 700-bp uniformly ^{32}P-labeled cDNA probe (specific activity = 4×10^8 cpm/μg). Autoradiography was for 72 hr with one intensifying screen at −70°C. 28S and 18S rRNAs are indicated.

been shown to be reversibly regulated by mitogens during myoblast growth in tissue culture (23). To determine whether JCC mRNA expression might be regulated in a manner similar to that of other genes involved in expressing the contractile phenotype, blot hybridizations were performed with the JCC cDNA probe CRC3 (700 bp) using RNA isolated from C2 and L_6E_9 (myogenic cell lines) and a nonfusing myoblast line, BC_3H1, under a variety of growth conditions. In both the C2 and L_6E_9 differentiated cells, the signal for JCC mRNA was barely detectable. However, differentiated BC_3H1 cells contained easily detectable levels of JCC mRNA (Figs. 4 and 5). Thus the BC_3H1 cells were chosen for further investigation. As shown in Fig. 4 (lane 1), growing subconfluent BC_3H1 cells did not contain detectable levels of JCC mRNA. However, within 24 hr after switching subconfluent cells to serum-deprived medium (0.5% FCS), there was a marked induction of JCC mRNA (lanes 3–5). The doublet seen in Fig. 4, lanes 3–5, may represent RNA cleavage and is not present in other RNA blots probed with the CRC cDNA (Figs. 2 and 4). JCC mRNA was also induced when BC_3H1 cells in growth medium reached confluence (data not shown). The expression of the JCC gene in BC_3H1 cells was reversible. As shown in Fig. 5*A*, when confluent BC_3H1 cells (lane 2) were replated at low density in growth medium, there was a marked decrease in the levels of JCC mRNA beginning at 24 hr after replating (lanes 3–5). A similar decrease in mRNA levels was seen when subconfluent induced (by serum deprivation) BC_3H1 cells were reexposed to 20% FCS (Fig. 5*B*, lanes 2–5).

DISCUSSION

The cloning and partial sequence of the subunit protein of the JCC of the SR are described. The cDNA probes obtained were used to identify a ≈16-kb mRNA in skeletal, heart, and smooth (aorta) muscle by Northern blot analysis, to estimate the molecular mass of the JCC subunit, and to study the expression of the mRNA during differentiation in a myoblast line (BC_3H1)

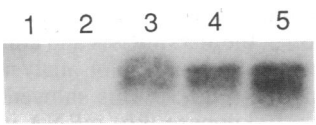

FIG. 4. Induction of JCC mRNA in BC_3H1 cells. RNA blot of BC_3H1 RNA hybridized with a 700-bp JCC cDNA probe. BC_3H1 cultures were plated at low density and grown in DMEM/20% FCS (lane 1). Cultures were then switched to low-serum medium for 10, 24, 48, and 72 hr (lanes 2–5, respectively). Only the relevant portion of the gel is shown; no other bands were present.

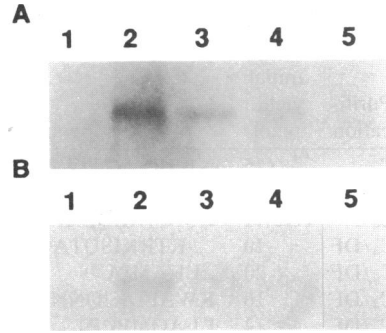

FIG. 5. Deinduction of JCC mRNA in BC_3H1 cells. RNA blot of BC_3H1 RNA hybridized with a 700-bp JCC cDNA probe. (*A*) BC_3H1 cultures grown in DMEM/20% FCS for 2 (lane 1) and 5 days (lane 2). Confluent cells (day 5) were replated and harvested at 24, 48, and 72 hr (lanes 3–5, respectively). (*B*) Subconfluent cultures plated at low density (lane 1) were induced by growth in low-serum medium for 48 hr (lane 2). Cultures were then switched to DMEM/20% FCS for 24, 48, and 72 hr (lanes 3–5, respectively). Only the relevant portion of the gel is shown; no other bands were present.

The ≈16-kb mRNA identified for the JCC in skeletal, heart, and aortic muscle from rabbit shows much weaker hybridization in heart and aorta then in skeletal muscle. Possible explanations for this observation are either that there is less SR and hence less JCC in heart and aortic muscle or that there is weak hybridization of the probes to these mRNAs due to sequence differences.

The JCC has been isolated from dog heart (17, 18, 24) as well as from skeletal muscle. The cardiac JCC has a slightly lower molecular mass by SDS/PAGE than the skeletal muscle JCC. There are also differences in immunoreactivity between the two channels (M.I. and S.F., unpublished observations) and differences in sensitivity to calcium activation (25). These findings would suggest that the cardiac and skeletal muscle JCCs have some structural differences.

The size of the JCC mRNA predicts a molecular weight of ≈550,000 (estimating ≈10% noncoding sequence for the mRNA). This would yield a molecular mass of ≈2200 kDa for the foot structure of the SR, assuming that the structure is composed of a tetramer as suggested by its fourfold symmetry (14, 15). This value is in close agreement with the observed mass of 2.3 ± 0.3 million obtained by scanning transmission electron microscopy (16).

The large size of the JCC has led to speculation as to the structure of the molecule. The JCC is considerably larger than other molecules whose primary function is to serve as ion-selective channels. For example, the ion-selective sodium and calcium channels are formed by multiple subunits, the largest of which is a polypeptide of <170 (α_1 subunit of the dihydropyridine receptor) or 260 kDa (for the α subunit of the sodium channel) (26). From electron micrographs it is known that much of the JCC molecule is an extra-membrane structure that accounts for the foot structures spanning the gap between the terminal cisternae of the SR and the T-tubule (8, 9, 15).

To investigate the possibility that there is coordinate regulation of the genes encoding the contractile proteins and those controlling calcium release, we have studied the expression of the JCC mRNA in C2, L_6E_9, and BC_3H1 cells. The mouse BC_3H1 cell line (23) has been used as a model for studying muscle cell differentiation and the regulation of muscle-related proteins. Removal of growth factors from the

culture medium or allowing cultures to reach confluence induces BC_3H1 cells to differentiate (27). During differentiation BC_3H1 cells accumulate muscle-specific gene products such as creatine phosphokinase (23, 27, 28), myokinase (27), the sarcomeric muscle isoforms of troponin T, the smooth and sarcomeric muscle isoforms of α-actin (29, 30), α-tropomyosin and myosin heavy and light chains (30), as well as the nicotinic acetylcholine (23) and insulin (31) receptors. In addition, it has been shown that BC_3H1 cells express a skeletal muscle-type voltage-gated Ca^{2+} channel (32). Differentiation is reversible either by adding mitogens to the culture medium (33) or by replating cells in growth medium (23, 28).

The time course for induction of JCC mRNA (Fig. 4) is similar to that reported for the induction of the contractile protein mRNAs (30). When undifferentiated cultures were plated at low density and switched from growth medium to low-serum medium, there was a striking increase in the expression of JCC mRNA by 48 hr (Fig. 4, lane 3). Moreover, the induction of JCC mRNA was reversible. When differentiated cultures were replated in growth medium, there was a rapid deinduction of the JCC mRNA (Fig. 5*A*, lanes 2–5). Similarly, when serum-deprived, subconfluent, differentiated cultures were switched to growth medium, JCC mRNA levels were significantly decreased within 24 hr and were absent by 48 hr (Fig. 5*B*, lanes 3–5). The time course for the deinduction of the JCC mRNA is also similar to that reported for the induction of a number of the contractile protein mRNAs (30).

Our data suggest that there is a common regulatory mechanism controlling the expression of the contractile proteins and the JCC of the SR. The availability, now, of molecular probes for the expression of the JCC should allow further dissection of the factors governing the differentiation of the components involved in the expression of the contractile phenotype.

Note. While this paper was under review Takeshima *et al.* (34) published the isolation and complete nucleotide sequence of the ryanodine receptor. Our sequence is identical to that of Takeshima *et al.* (34). They also show that the translation product of the cloned cDNA binds ryanodine, demonstrating that this sequence corresponds to the ryanodine receptor.

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