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Structure of the Rabbit Fast-twitch Skeletal Muscle Calsequestrin Gene*

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Angel Zarain-Herzberg‡, Larry Fliegel§, and David H. MacLennan¶

From the Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario, Canada, M5G 1L6

We have isolated two overlapping 17-kilobase (kb) genomic clones which, together, encode the rabbit fast-twitch skeletal muscle calsequestrin. The calsequestrin gene is 8.6 kb in length and consists of 11 exons. S1 nuclease protection and primer extension analyses position the transcription initiation site 191 nucleotides upstream of the translation initiation codon. The 5′-flanking sequence contains a CCAAT sequence and two CG-rich sequences but does not have an identifiable TATA box sequence in the anticipated position just upstream of the transcription initiation site.

The sequence of the calsequestrin gene confirmed the sequence deduced from cDNA clones derived from neonatal muscle mRNA (Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A. F., and MacLennan, D. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1167-1171). We could not detect any potential alternative splicing of the transcript in an analysis of this region of the gene that would account for the different sequence found at the carboxyl terminus of adult rabbit fast-twitch skeletal muscle calsequestrin.

Calsequestrin is a major protein in the sarcoplasmic reticulum. It is localized in the lumen of the terminal cisternae in mammalian (1-6), amphibian (7), and avian skeletal muscles (8), and in mammalian cardiac muscle (9, 10). Since calsequestrin binds Ca^{2+} with high-capacity (1 μmol of Ca^{2+}/mg of protein) and with moderate affinity (K_d of about 1 mM) it undoubtedly functions as a Ca^{2+} storage protein in the lumen of the sarcoplasmic reticulum (1, 11-15). Partial amino acid sequence has been obtained for the adult protein (6, 16), and the complete amino acid sequence has been deduced from a cDNA clone encoding calsequestrin in neonatal rabbit fast-twitch skeletal muscle (17). The cDNA sequence encodes a protein of 367 residues plus a 28-residue amino-terminal signal sequence. The high capacity Ca^{2+} binding in the protein can be explained on the basis of the amino acid sequence which is extremely acidic, especially in the carboxyl-terminal region. The deduced amino acid sequence from neonatal cDNA and the partial amino acid sequence correspond except for the carboxyl terminus where an extension of seven aspartic acids was found in the deduced sequence. This suggests that the neonatal and adult forms of calsequestrin could differ as a result of alternative splicing (17), as is the case for the fast-twitch Ca^{2+}-ATPase (18-20). In this paper we describe the cloning and characterization of the gene that encodes the fast-twitch skeletal muscle form of calsequestrin and demonstrate that alternative splicing is unlikely for this gene.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Bethesda Research Laboratories, and New England Biolabs. Oligo(dT)-Sepharose was from Collaborative Research. The plasmids pTZ 18R and 19R were from Pharmacia LKB Biotechnology Inc. [γ-^{32}P]ATP was obtained from ICN, and [α-^{32}P]-dCTP was purchased from Amersham Corp. All other chemicals were of the highest grade available.

Isolation of Genomic Clones—A rabbit genomic library constructed by partial *Hae*III and *Alu*I digestion of liver DNA and cloning into λ phage Charon 4A (21) was a generous gift from Dr. T. Maniatis, Harvard University. The genomic library was screened by hybridization (22) with a mixture of two nick-translated fragments isolated from the 5′ or 3′ ends of calsequestrin cDNA. These included a 260 bp[1] *Pst*I-*Pvu*II fragment containing the G:C tail and the 5′ end of a neonatal cDNA clone and a 387-bp *Eco*RI-*Sst*I fragment containing the 3′ end of the same cDNA clone (17). Genomic DNA clones in λ phage Charon 4A were isolated as described by Maniatis *et al.* (22). They were mapped with restriction endonucleases and fragments obtained from the digests were subcloned into pTZ 18R and 19R.

DNA Sequencing—Single stranded DNA templates of the individual subfragments cloned into PTZ 18R and 19R were sequenced by the dideoxy method of Sanger *et al.* (23).

Southern Blot Analysis—Genomic DNA clones were digested with restriction endonucleases, separated by electrophoresis in 0.8% agarose gel, strained with ethidium bromide, and blotted onto nitrocellulose (24). Hybridization with ^{32}P-labeled cDNA fragments was performed according to the method of Nilson *et al.* (25). For hybridization with 17 base synthetic oligonucleotides, the oligonucleotides were labeled at the 5′ end to a specific activity of 1×10^9 dpm/μg with [γ-^{32}P]ATP and T4 polynucleotide kinase. Genomic DNA blots were prehybridized for 6 h in 50 ml of 2 × SSCP, pH 7.0, 10 × Denhardt's solution, and 0.1 mg/ml poly(A)$^+$ (Boehringer Mannheim) at 22 °C. Hybridization was carried out overnight in the same solution in the presence of 2 ng/ml of ^{32}P-labeled oligonucleotide probes. The filter was washed twice in 2 × SSCP at 37 °C for 30 min and twice in 1 × SSCP at 37 °C for 30 min. It was then dried onto Whatman No. 3 MM paper at 22 °C and exposed to Kodak XAR-5 film for 6 h.

RNA Isolation—Total RNA and poly(A)$^+$ RNA were isolated from rabbit fast-twitch skeletal muscle (psoas) during different developmental stages: late fetal (28 days of gestation), neonatal (3 and 8

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03181.

‡ Postdoctoral Fellow of the Charles H. Best Foundation. Present address: Dept. of Physiology and Biophysics, College of Medicine, University of Vermont, Burlington, VT 05405.

§ Postdoctoral Fellow of the Heart and Stroke Foundation of Ontario.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: bp, base pairs; SSCP, a solution of 120 mM sodium chloride, 15 mM sodium citrate, 13 mM potassium phosphate, and 1 mM disodium EDTA, pH 7.2; kb, kilobase.

days), and adult (365 days), according to the procedure described previously (26).

S1 Nuclease Mapping—A 650-bp *Pst*I-*Rsa*I genomic DNA fragment covering the putative transcription initiation site (see Fig. 1) was subcloned in pTZ 18R and a ^{32}P-labeled single-stranded probe, complementary to calsequestrin mRNA, was isolated by the procedure of Ley *et al.* (27), modified for use with pTZ. Briefly, the 17-base reverse M13 primer (Pharmacia LKB Biotechnology Inc.) was annealed at 65 °C for 10 min with the single-stranded template, and synthesis of the radiolabeled second strand was carried out in the presence of [α-^{32}P]dCTP (3000 Ci/mmol) by the large fragment of DNA polymerase I at 37 °C for 20 min. The double-stranded complex was cleaved with *Hin*dIII within the pTZ polylinker, and the cleaved strands were separated by heat denaturation and polyacrylamide/urea gel electrophoresis. A radiolabeled, single-stranded fragment of 725 bases that included the polylinker and the primer sequence of pTZ was identified by autoradiography and eluted from the gel.

S1 nuclease protection of the 5′ end of the calsequestrin gene transcript using mRNA isolated from rabbit fast-twitch skeletal muscle at different developmental stages was assayed using a modification of the method of Berk and Sharp (28). Fast-twitch skeletal muscle total RNA (5–10 μg) or poly(A)$^+$ RNA (10 μg) were mixed with 7×10^5 dpm of probe in 50 μl of a solution comprised of 80% deionized formamide, 0.4 M NaCl, 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 0.05% sodium dodecyl sulfate. The hybridization mixture was incubated first at 75 °C for 15 min and then at 50 °C for 12 h. The nucleic acids were incubated with 200 units of S1 nuclease (Boehringer Mannheim) in 350 μl of a solution containing 200 mM NaCl, 30 mM sodium acetate, pH 4.6, and 3 mM $ZnSO_4$ at 37 °C for 1 h to digest single strands. The samples were ethanol precipitated, and the pellets were dissolved in loading buffer (90% formamide, 0.8 mM EDTA, 1% xylene cyanol, 0.1% bromphenol blue), boiled for 2 min, quick-cooled on ice, and analyzed on a 5% polyacrylamide-sequencing gel containing 8 M urea. Radioactive bands were identified by autoradiography with Kodak XAR-5 film, and their sizes were estimated by comparison with nucleic acid oligomeric standards.

Primer Extension Analysis—Two 17-base synthetic oligonucleotide primers, complementary to sequences located at position −155 to −171 bp (primer 1) and −82 to −98 bp (primer 2) upstream of the calsequestrin translation initiation codon in the fast-twitch muscle mRNA (see Fig. 1) were synthesized. They were 5′ end-labeled to a specific activity of $1–3 \times 10^9$ dpm/μg with [γ-^{32}P]ATP and T4 polynucleotide kinase at 37 °C for 1 h. Unincorporated [γ-^{32}P]ATP was removed by binding to a NACS.52 Pre-Pac column (Bethesda Research Laboratories) in 100 mM NaCl, 10 mM Tris/HCl, pH 7.4, and 1 mM EDTA. One pmol of primer (1.2×10^7 dpm) was hybridized to total RNA (10 μg) or to poly(A)$^+$ RNA (10 μg) in 30 μl of a solution containing 100 mM NaCl and 10 mM Tris/HCl, pH 7.4. Mixtures were heated at 75 °C for 2 min and transferred to 37 °C for 30 min. Twenty μl of 5 × reverse transcription buffer (250 mM Tris/HCl, pH 8.3, 250 mM KCl, 40 mM $MgCl_2$, and 40 mM dithiothreitol), 10 μl of a mixture of all deoxynucleotide triphosphates (10 mM each) and 40 μl of H_2O were then added. Synthesis of cDNA was started by the addition of 200 units of M-MLV reverse transcriptase (Bethesda Research Laboratories) and incubation was at 37 °C for 1 h. The reaction mixture was ethanol precipitated, and the pellet was resuspended in loading buffer, heat denaturated, and analyzed on a 7.5% polyacrylamide/urea gel as described above for S1 nuclease mapping.

RESULTS

Structure of the Rabbit Calsequestrin Gene—About 1×10^6 recombinant phages from a rabbit genomic library constructed in λ phage Charon 4A (21), were screened using ^{32}P-labeled rabbit fast-twitch skeletal muscle calsequestrin cDNA fragments as probes. Eight positive recombinant phages were obtained, six with the 260-bp *Pst*I-*Pvu*II probe from the 5′ end of the cDNA and two with the 387-bp *Eco*RI-*Sst*I probe from the 3′ end of the cDNA. They were subjected to further analysis by restriction enzyme mapping and Southern blotting (24). The genomic DNA inserts of about 17–17.3 kb in length were found in two groups of clones labeled λ RbFC1 and λ RbFC2 (see Fig. 2). The inserts in these clones overlapped for about 2.7 kb. Fig. 2 shows the restriction maps of λ RbFC1 and λ RbFC2 as well as the exon/intron organization of the rabbit fast-twitch calsequestrin gene deduced from comparison of the nucleotide sequence of the cDNA (17) with the genomic clone sequences. Eleven exons distributed over about 8.6 kb accounted for the full-length fast-twitch calsequestrin transcript.

Fig. 1 presents the nucleotide sequence of the rabbit fast-twitch calsequestrin gene. Exon 1 was 452 bp long and contained all of the 5′-untranslated sequence plus the coding sequence for the 28-amino acid signal peptide and 59 amino acids at the amino terminus of the mature protein. Exon 11 was 637 bp long and encoded the carboxyl-terminal 48 amino acids plus the complete 3′-untranslated sequence ending in the polyadenylation site. The nucleotide sequences observed at all 5′ donor and 3′ acceptor splice sites were in agreement with the consensus sequence /gt—ag/ described previously (29, 30). A tract of polypyrimidines preceded all intron/exon boundaries.

Location of the Transcription Initiation Site and Analysis of Upstream Sequences—In order to identify the cap site in calsequestrin mRNA, primer extension and S1 nuclease protection analyses were performed. Primer extension experiments using total RNA or poly(A)$^+$ RNA isolated from adult rabbit fast-twitch skeletal muscle are shown in Fig. 3. A major extension product of 37 bases was obtained upon reverse transcription with primer 1. A major extension product of 110 bases was obtained with primer 2. These experiments locate the transcription initiation site at a position 191 bp upstream of the translation initiation codon AUG. Thus, the calsequestrin cDNA clone described in Ref. 17 was only 21 bp short of a full length transcript.

In order to confirm the results obtained with primer extension, S1 nuclease protection assays were performed with total RNA or poly(A)$^+$ RNA isolated from rabbit fast-twitch skeletal muscle at different stages of development. As shown in Fig. 4, a single band of 138 ± 3 bases was protected in total RNA from late fetal (28 days of gestation), neonatal (3 and 8 days), and adult (1 year) fast-twitch skeletal muscle. This experiment also located the transcription initiation site at a position 188–194 nucleotides upstream from the translation initiation site. Both techniques indicate that the adenosine at a position 191 bases upstream of the initiation codon is the probable cap site at each of these stages of development.

The nucleotide sequence immediately upstream of the cap site in the calsequestrin gene (see Fig. 1) contained a CCAAT consensus sequence (31) located 58 bp upstream of the cap site. A "TATA" box sequence (29) was missing from its usual position at about 30 bp upstream of the cap site, but the sequence CCGCCC (32–35) was found 28 bp upstream of the cap site and again at a position 158 bp upstream of the cap site. The sequence CATAT that could serve as a variant TATA box was observed 93 bp upstream of the cap site and 35 bp upstream of the CCAAT sequence.

Analysis of the Potential for Alternative Splicing in the Calsequestrin Gene—The carboxyl terminus determined by amino acid sequences analysis of mature calsequestrin differed from the carboxyl terminus deduced from a cDNA clone constructed from mRNA of neonatal muscle (17). In order to determine whether developmental changes occurred in calsequestrin gene expression, calsequestrin clones were also isolated from a cDNA library made from mRNA isolated from 1-year-old fast-twitch muscle (19). The restriction maps of this group of clones and the DNA sequences of these clones were found to be identical at their 3′ ends to the clone isolated from neonatal muscle (not shown).

Exon 11 was found to be a very long exon which covered the region in which alternative splicing might take place (Figs. 1 and 2). The sequence of the exon was identical to the

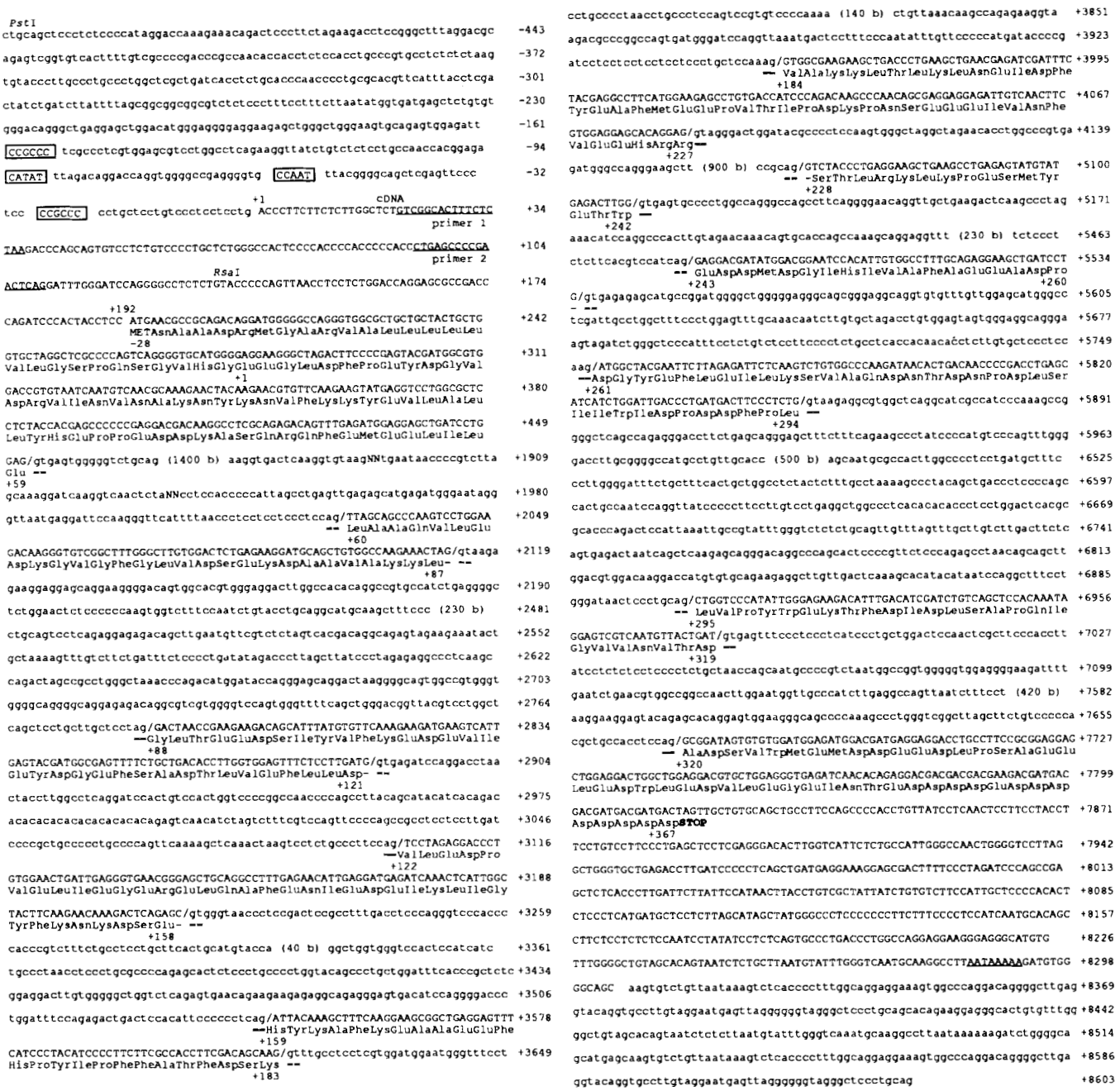

FIG. 1. **Nucleotide sequence of the rabbit fast-twitch calsequestrin gene.** DNA sequences corresponding to exons are in *capital letters*; intron sequences are in *lower case letters*. Nucleotide position +1 is assigned to the transcription initiation site (cap site) as determined by S1 nuclease mapping and primer extension analyses (see Figs. 4 and 5) and by cDNA sequence analysis. The last nucleotide in every line is *numbered* on the *right*. The approximate lengths of unsequenced gaps are indicated, and the numbering system incorporates the lengths of these gaps. The number of each amino acid in the mature protein is indicated in the line below the amino acid sequence beginning with the amino-terminal Glu residue (+1). The amino acids constituting the signal peptide sequence are *numbered negatively* so that the initiator methionine is assigned to position (−28). The CCAAT sequence, two C-G boxes and the sequence CATAT which is upstream of the CCAAT box sequence are *boxed*. The polyadenylation signal (AATAAAAA) and the nucleotide regions complementary to the 17 base synthetic oligonucleotides used for primer extension analyses (primer 1 and 2) are *underlined*. The exon/intron and intron/exon boundaries are indicated by a *diagonal line*. The gt and ag residues present at the 5′ and 3′ boundaries of introns are *doubly underlined*. The restriction endonucleases (*Pst*I and *Rsa*I) used to generate the 650 base probe used for S1 mapping are indicated above the nucleotide sequence. The 5′ end of our neonatal calsequestrin cDNA is indicated by the *A* in *cDNA* above the nucleotide sequence.

sequence published for the 3′ end of neonatal calsequestrin cDNA (17) and found for adult calsequestrin cDNA (not shown). However, it was conceivable that a second complete exon existed 3′ to exon 11 and encoded the adult form of the protein. Accordingly, the genomic insert in λ RbFC2 including the 9.5-kb segment of genomic DNA that extended from the 3′ end of exon 11 to the insertion site was probed to determine whether it harbored a second 3′ exon. Because both the neonatal and adult sequences were found to contain a sequence of at least 5 Asp residues (neonatal sequence -Glu-

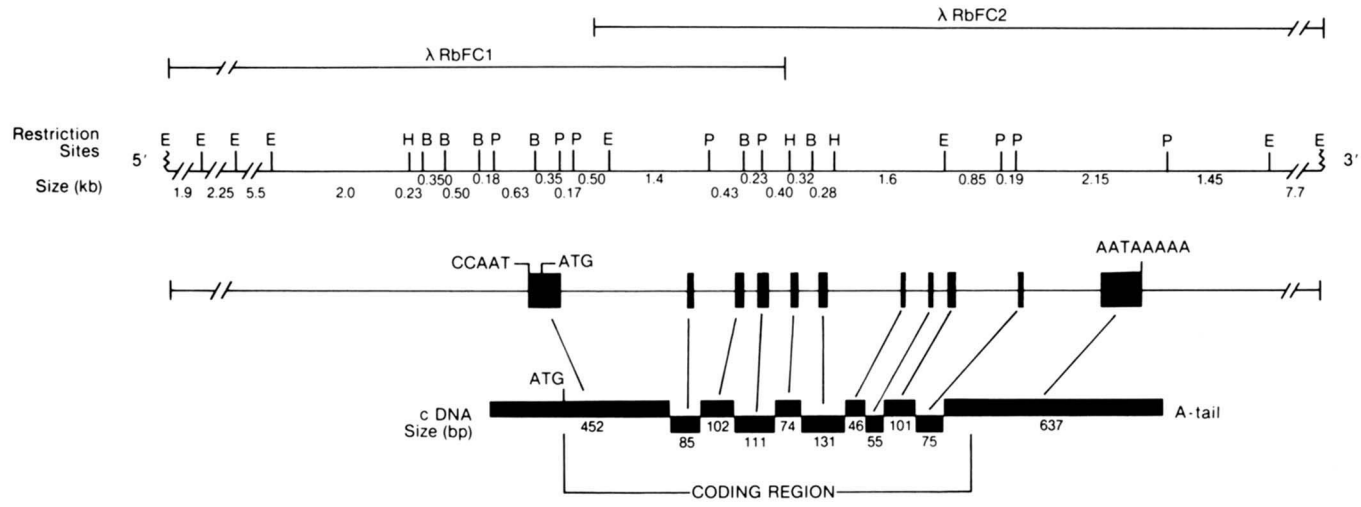

FIG. 2. **Structural analysis of the rabbit fast-twitch calsequestrin genomic clones.** The two upper lines represent the location of the overlapping genomic clones RbFC1 (17 kb) and λ RbFC2 (17.3 kb) spanning a total length of 31.7 kb of genomic DNA. The third line shows the partial restriction endonuclease map of the two overlapping genomic clones. Restriction endonuclease sites: *E, Eco*RI; *B, Bam*HI; *H, Hin*dIII; *P, Pst*I. Artificial *Eco*RI sites generated during the cloning procedure are marked by a *jagged line.* The fourth line shows the locations of the 11 exons within the gene as *solid boxes.* The positions of the CCAAT sequence, the ATG initiation codon and the polyadenylation signal (AATAAAAA) are marked by *vertical lines.* The bottom line represents the exon boundaries within a neonatal fast-twitch calsequestrin cDNA clone (17).

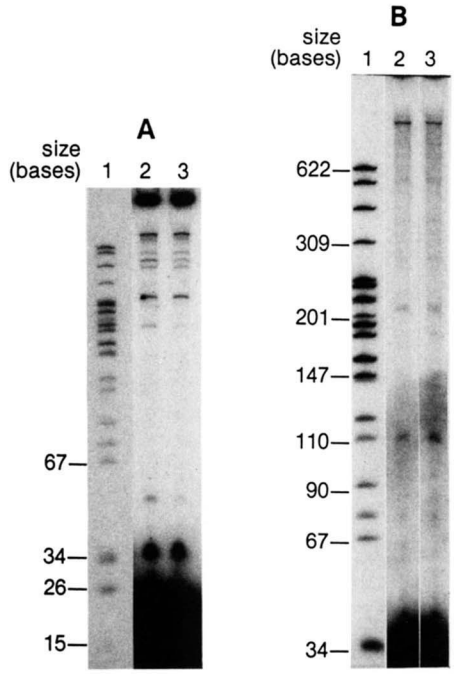

FIG. 3. **Primer extension analysis of the 5′ end of the rabbit fast-twitch calsequestrin mRNA.** Two 17-base ^{32}P-labeled synthetic oligonucleotides (primer 1 and 2, see Fig. 1), complementary to the 5′-untranslated region of the fast-twitch calsequestrin mRNA, were annealed to 10 μg of total RNA or 10 μg of poly(A)$^+$ RNA from adult fast-twitch skeletal muscle at 75 °C and primer extended with M-MLV reverse transcriptase at 37 °C as described under "Experimental Procedures." The products were analyzed by electrophoresis on a 7.5% polyacrylamide/urea gel. *Panel A* shows the products extended with primer 1, utilizing total RNA (*lane 2*) and poly(A)$^+$ RNA (*lane 3*). *Panel B* shows the products extended with primer 2 using total RNA (*lane 2*) and poly(A)$^+$ RNA (*lane 3*). *Lane 1* in both panels contains ^{32}P-labeled *Hpa*II fragments of pBR322 DNA as size markers.

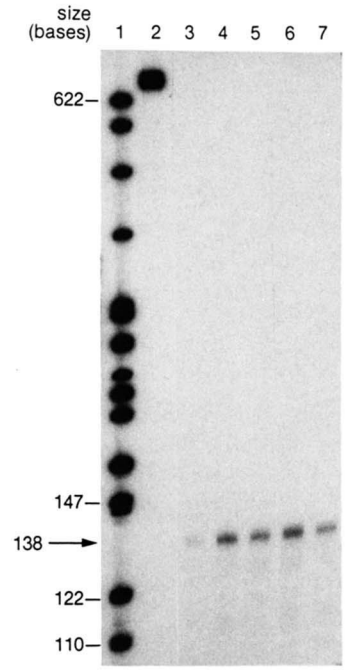

FIG. 4. **S1 nuclease mapping of the 5′ end of rabbit fast-twitch skeletal muscle calsequestrin mRNA.** The single-stranded ^{32}P-labeled 650-base *Pst*I-*Rsa*I fragment from clone λ RbFC1 (see Fig. 1) was annealed to 5–10 μg of total RNA or poly(A)$^+$ RNA isolated from fast-twitch skeletal muscle during different developmental stages. S1 nuclease protection assays were performed as described under "Experimental Procedures." The protected DNA-RNA hybrids were analyzed in 5% polyacrylamide/urea gels. ^{32}P-Labeled *Hpa*II fragments of pBR322 were used as size markers. *Lane 1*, size markers; *lane 2*, undigested probe 725 bases long; *lane 3*, total RNA (5 μg) from adult skeletal muscle; *lane 4*, poly(A)$^+$ (10 μg) RNA from adult skeletal muscle; *lane 5*, total RNA (10 μg) from 28-day-old fetal muscle; *lane 6*, total RNA (10 μg) from 3-day-old muscle; *lane 7*, total RNA (10 μg) from 8-day-old muscle.

$(Asp)_4$-Glu$(Asp)_8$; adult sequence -Glu-$(Asp)_5$-Glu), a probe was constructed which would bind to cDNA sequences encoding polyaspartate. The synthetic oligonucleotide 5′-TCPuTCPuTCPuTCPuTCPuTC-3′ was labeled with ^{32}P and used to probe restriction fragments of λ RbFC2 for the presence of this sequence (Fig. 5). Digestion with *Eco*RI, *Eco*RI plus *Pst*I,

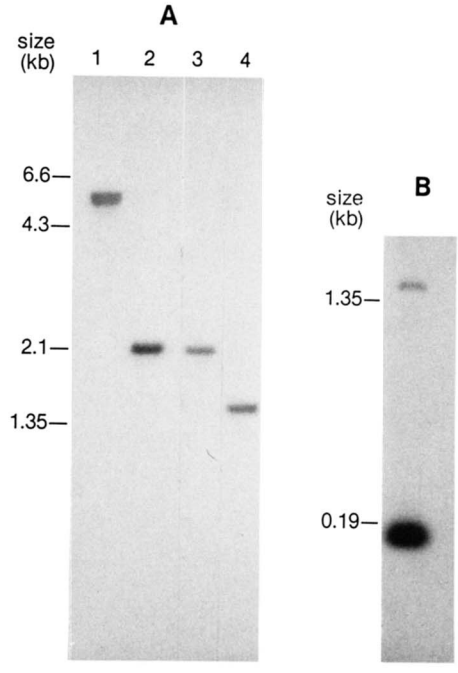

FIG. 5. **Southern blotting of restriction fragments of clone λ RBFC2 with oligonucleotides complementary to sequences encoding polyaspartate.** *Panel A* represents a Southern blot in which clone λ RBFC2 was digested with *Eco*RI (*lane 1*), *Pst*I (*lane 2*), *Eco*RI + *Pst*I (*lane 3*), and *Pst*I + *Xho*I (*lane 4*) and probed with the sequence 5′-TCPuTCPuTCPuTCPuTC-3′. *Panel B* represents a Southern blot of a subcloned 1.35-kb *Pst*I-*Xho*I fragment from clone λ RbFC2, digested with *Pst*I + *Xho*I + *Sst*II. The blots were hybridized with a mixture of 5′ ^{32}P-labeled 17-base oligonucleotide probes complementary to the sequence -(Asp)$_5$-Glu- based on the carboxyl-terminal sequence determined for adult calsequestrin (5, 16). The conditions for hybridization were as described under "Experimental Procedures."

*Pst*I plus *Xho*I, and *Pst*I plus *Xho*I plus *Sst*II yielded only a single hybridizing fragment which, in the latter digestion, was an *Xho*I-*Sst*II fragment 173 bp long.

DISCUSSION

The gene encoding rabbit fast-twitch skeletal muscle calsequestrin is relatively small and uncomplicated. It is only 8.6 kb long and contains 11 exons. The exon/intron boundaries within the coding region interrupt sequences encoding amino acids predicted to be located in turns or at the beginning or ends of α-helices in 9 of 10 cases. Only intron 1 disrupts DNA encoding an amino acid sequence strongly predicted to form an α-helix (17). Amino acid sequences which are strongly predicted to form β-sheet structures (17) are each encoded inside of exons.

The region upstream of the 5′ end of the gene is atypical in that it does not contain a TATA box immediately upstream of the cap site. The TATA box sequence, however, has been shown to be absent from the 5′-flanking region of a number of mammalian housekeeping genes or to be located far upstream of the cap site (36–39). Deletion of this sequence from the H2A histone gene (40) did not abolish transcription but increased the heterogeneity at the 5′ end of the transcript, suggesting that the TATA motif is a specificity element. The CCAAT box sequence at the 5′ end of the gene might bind the transcription factor CTF (41) which can act both as a selectivity factor and as an initiation factor. Potential regulatory sequences such as the two CG boxes (32–35) are found in the 5′-flanking sequence and may function as promoter elements in the calsequestrin gene.

The 5′ sequences upstream of the transcription initiation sites for calsequestrin and the Ca^{2+}-ATPase (12) differ in many respects. Both have the CCAAT sequence but only the Ca^{2+}-ATPase has a TATA box sequence in the proper position. The calsequestrin gene has two CG boxes, but the Ca^{2+}-ATPase has none. Thus, one would expect these two genes to be transcribed differently. This is, indeed, the case, since we have shown previously (42–43) that calsequestrin synthesis is initiated much earlier in rat muscle cells in culture than is the Ca^{2+}-ATPase.

Analysis of Alternative Splicing—The amino acid sequence of adult calsequestrin was found to end in Glu-(Asp)$_5$-Glu while the comparable carboxyl-terminal sequence deduced from cDNA was Glu-(Asp)$_4$-Glu-(Asp)$_8$ (17). In our initial interpretation of these results (17), we suggested that calsequestrin transcripts might be alternatively spliced, giving rise to the more acidic form in neonatal life and to the less acidic form in adult life. Our analysis of the adult transcripts does not support this theory since no difference was detected between these two. Our analysis of the gene also suggests that alternative splicing is unlikely. The position where divergence in the sequences occurs lies well within a large exon of 637 bp. Thus, alternatively spliced exons would have to encode a stretch of 40 identical amino acid residues before diverging. No alternative sequence of this degree of identity was found in the 9.5 kb downstream of exon 11. It is conceivable that such a sequence occurs further downstream of a 9.5 kb intron, but this also seems unlikely.

Three explanations would reconcile our findings. The first is that the amino acid sequence was incorrect, the second is that there is post-translational modification of calsequestrin (perhaps proteolytic cleavage), and the third is that the additional carboxyl-terminal acidic residues are lost during isolation of the protein. Sequencing studies (44) could not distinguish accurately between the sequences -Glu-(Asp)$_4$-Glu and -Glu-(Asp)$_5$-Glu because of repetitive Asp residues, and we conclude that the DNA sequence -Glu-(Asp)$_4$-Glu is correct. No amino acids were found after glutamate by automated Edman degradation of the carboxyl-terminal, cyanogen bromide-derived peptide (44). Reinvestigation of the amino acid sequence of the carboxyl terminus of calsequestrin is required.

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