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Complementary DNAs were isolated and used to deduce the primary structures of the α_1 and α_2 subunits of the dihydropyridine-sensitive, voltage-dependent calcium channel from rabbit skeletal muscle. The α_1 subunit, which contains putative binding sites for calcium antagonists, is a hydrophobic protein with a sequence that is consistent with multiple transmembrane domains and shows structural and sequence homology with other voltage-dependent ion channels. In contrast, the α_2 subunit is a hydrophilic protein without homology to other known protein sequences. Nucleic acid hybridization studies suggest that the α_1 and α_2 subunit mRNAs are expressed differentially in a tissue-specific manner and that there is a family of genes encoding additional calcium channel subtypes.

THE L-TYPE BUT NOT THE T-TYPE OR N-type Ca^{2+} channels are sensitive to "Ca²⁺ antagonist" drugs, including the dihydropyridines (DHPs). Skeletal muscle DHP-sensitive Ca^{2+} channels (L-type) comprise at least two large polypeptide subunits, α_1 and α_2 , which copurify with equimolar stoichiometry (2-5). The α_1 subunit, which may be weakly glycosylated, contains receptor sites for at least two classes of Ca^{2+} antagonists, the dihydropyridines and the phenylalkylamines, and has a molecular mass of 155 to 170 kD under both reducing and nonreducing conditions. The α_2 subunit, which is extensively glycosylated, does not bind either DHP or phenylalkylamine Ca^{2+} antagonists and has a molecular mass of 165 to 175 kD under nonreducing conditions and of 135 to 150 kD under reducing conditions. Two additional subunits may also be present, a nonglycosylated β subunit (50 to 55 kD) and a glycosylated γ subunit (30 to 33 kD) (2, 3, 6, 7). In addition, the α_1 and β subunits are substrates for protein kinases, whereas the α_2 and γ subunits are not (7, 8). Although active DHP-sensitive Ca^{2+} channels have

been reconstituted in vitro (9), it is unknown which subunits are required for a functional DHP-sensitive Ca^{2+} channel.

We describe here the sequences and tissue-specific expression of the α_1 and α_2 subunits of a DHP-sensitive Ca^{2+} channel determined by using, cloned cDNA sequences. To isolate the cDNA clones, monoclonal antibody IIF7, specific for the α_1 subunit from rabbit skeletal muscle triads (6), and guinea pig polyclonal antisera, specific for the gel-purified α_2 subunit (4), were each used to screen 1.0×10^6 recombinant phage of a rabbit back skeletal muscle cDNA library. Overlapping cDNA clones were isolated to determine the DNA sequence encoding each subunit (Fig. 1).

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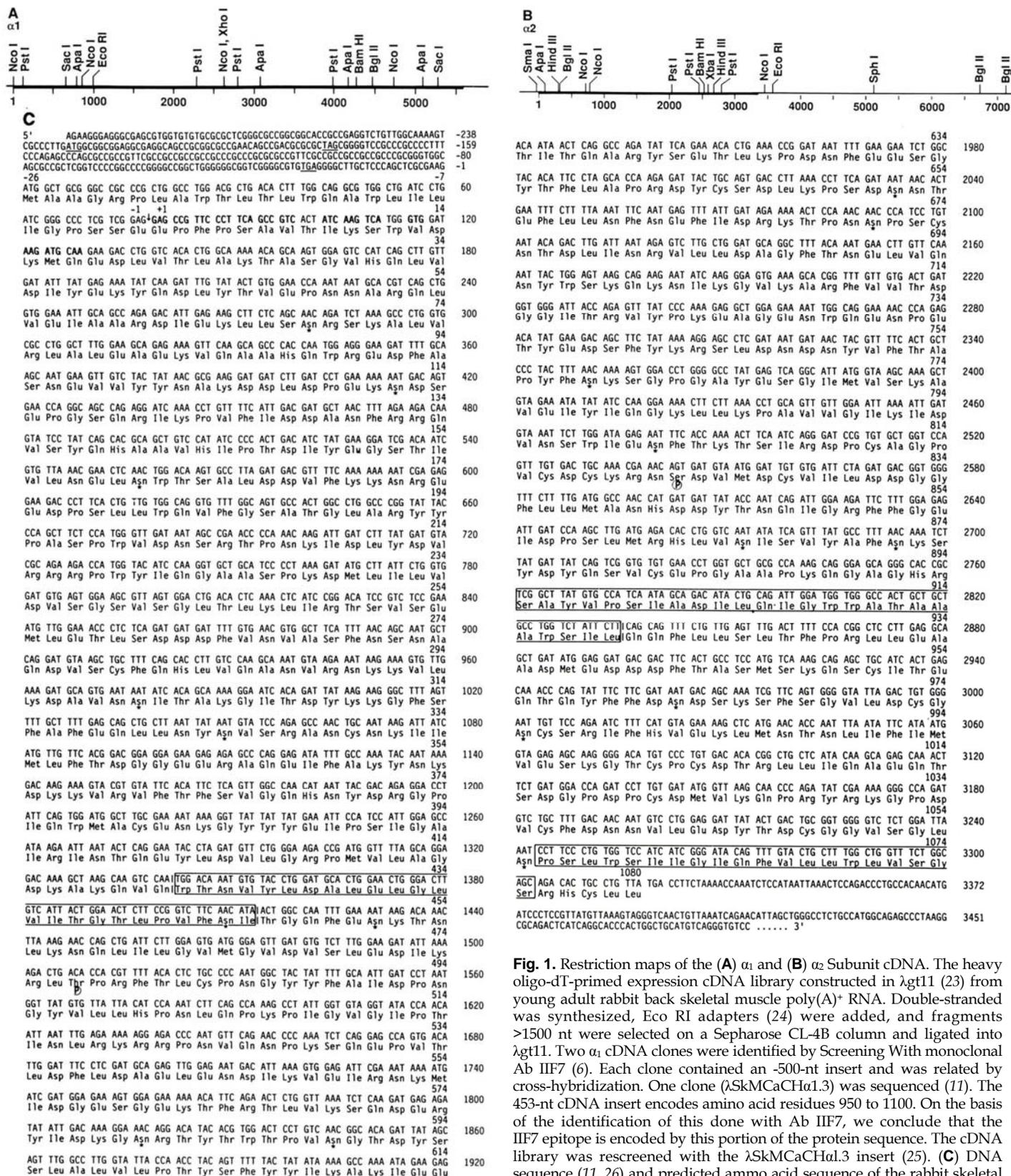


Fig. 1. Restriction maps of the (A) α_1 and (B) α_2 Subunit cDNA. The heavy oligo-dT-primed expression cDNA library constructed in λ gt11 (23) from young adult rabbit back skeletal muscle poly(A)⁺ RNA. Double-stranded was synthesized, Eco RI adapters (24) were added, and fragments >1500 nt were selected on a Sepharose CL-4B column and ligated into λ gt11. Two α_1 cDNA clones were identified by Screening With monoclonal Ab IIF7 (6). Each clone contained an -500-nt insert and was related by cross-hybridization. One clone (λ SkMCACh1.3) was sequenced (11). The 453-nt cDNA insert encodes amino acid residues 950 to 1100. On the basis of the identification of this done with Ab IIF7, we conclude that the IIF7 epitope is encoded by this portion of the protein sequence. The cDNA library was rescreened with the λ SkMCACh1.3 insert (25). (C) DNA sequence (11, 26) and predicted amino acid sequence of the rabbit skeletal muscle Ca²⁺ channel α_2 subunit. A presumptive amino acid signal peptide is shown as the first 26 amino acids (residues -26 to -1). An arrow identifies the proposed cleavage site and positive numbering starts with the proposed NH₂-terminal residue of the mature protein. The NH₂-terminal amino acid Sequence previously determined (4) is shown in bold sequence; Thr⁸, Trp¹², and Asp¹⁴ were not previously determined. An in-frame upstream stop codon is underlined, as well as the start and stop codons of an upstream short open reading frame. Three putative transmembrane regions are enclosed in boxes. Potential N-glycosylation and phosphorylation sites are identified by an asterisk (*) below Asn residues and P below the Ser and Thr residues, respectively. Three α_2 cDNA clones were identified by screening the λ gt11 rabbit back skeletal muscle cDNA library with guinea pig antisera to α_2 . Two clones, λ SkMCACh2.2 (2.5 kb) and λ SkMCACh2.4 (3.6 kb), overlapped to encode 4.75 kb of an ~8-kb transcript (Fig. 3B). An additional 14 overlapping clones encoding a total of ~7850 nt were isolated by DNA hybridization. Only 176 nt of ~4224 nt of 3' untranslated sequence was confirmed in both directions and are reported.

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The α_1 subunit cDNA contains a 5619-nucleotide (nt) open reading frame that encodes a sequence of 1873 amino acids (Fig. 1A). This cDNA sequence is consistent with an ~6500 nt DHP receptor α_1 mRNA (see Fig. 3A). Although the nucleotide sequence of this cDNA is 99.4% identical to the cDNA sequence of the DHP receptor reported by Tanabe *et al.* (10), a number of differences were determined. Nucleotide differences were identified at 33 positions (11), of which three result in amino acid changes. The amino acid differences occur at residues 1808 (Thr to Met), 1815 (Ala to Val), and 1835 (Ala to Glu).

We also cloned approximately 7850 nt of α_2 cDNA (Fig. 1B), which is consistent with an ~8000-nt α_2 mRNA (see Fig. 3B). Three hundred and eight nucleotides of 5' untranslated sequence, a 3318-nt open reading frame, and 176-nt of 3' untranslated sequence are shown in Fig. 1C. The 5' untranslated sequence of the α_2 cDNA is unusually long (12). The open reading frame encodes a sequence of 1106 amino acids (Fig. 1C). The deduced amino acid sequence yields a calculated molecular weight of 125,018 for the α_2 subunit, which contrasts with the observed molecular mass of 165 to 175 kD (under nonreducing conditions) determined by SDS-polyacrylamide gel electrophoresis (2, 3, 6, 7). However, the agreement between the sequence of amino acid residues 1 to 17 (Fig. 1C) with the partial amino-terminal sequence of the α_2 subunit (4) indicated that we cloned the α_2 subunit. We propose that the α_2 subunit has a 26-amino acid (residues -1 to -26) signal sequence. Although this proposed signal sequence is hydrophobic and of an appropriate characteristic length (13), it has an unusually short central hydrophobic region defined by Glu⁻¹ and Gln⁻¹². The α_2 subunit contains 18 potential *N*-glycosylation sites (14) and two potential adenosine 3',5'-monophosphate (cAMP)-dependent phosphorylation sites (15) (Fig. 1C).

The α_2 subunit lacks significant homology with any protein in the Dayhoff protein sequence database or with other sequences of ion channel and receptor proteins. An analysis of the α_2 subunit sequence for regional hydrophathy (16) reveals that, in contrast to the α_1 subunit, this protein is substantially hydrophilic, although it does contain a number of hydrophobic regions that may represent transmembrane domains (Fig. 2). These segments are designated I (amino acid residues 422 to 445), II (residues 895 to 919), and III (residues 1056 to 1075) in Figs. 1C and 2. If the proposed α_2 subunit signal sequence is, in fact, cleaved between the Glu⁻¹ and Glu⁺¹ residues (Fig. 1C), the amino terminus would be extracel-

lular. Furthermore, assuming that the three hydrophobic segments (Fig. 2) are transmembrane domains and that there are only three such domains, the carboxyl terminus would be intracellular. Such a transmembrane topography would result in 8 of the 18 potential *N*-glycosylation sites being extracellular and the two potential phosphorylation sites being intracellular (Fig. 2) and would be consistent with biochemical studies on the α_2 subunit (2, 3, 6-8).

To determine the tissue-specific expres-

sion of α_1 and α_2 mRNAs, total RNA was isolated from a variety of rabbit tissues for RNA blot analysis (Fig. 3). For the α_1 subunit, a prominent 6.5-kb transcript was detected in total RNA from skeletal muscle, while a much weaker hybridizing transcript of the same size was observed in aorta (Fig. 3A). Upon longer exposures of the autoradiograms, a weakly hybridizing 6.5-kb transcript was also observed in total RNA from heart; however, no α_1 -specific hybridization was observed with total RNA from ileum or

Fig. 2. Hydrophility profile of the α_2 subunit computed according to Kyte and Doolittle (16); the window size is 19 residues plotted at one-residue intervals. Potential glycosylation sites (*) and phosphorylation sites (P) are indicated. Three transmembrane domains are proposed: I, amino acid residues 422 to 445; II, amino acid residues 895 to 919; and III, amino acid residues 1056 to 1075. Transmembrane regions are proposed based on their hydrophathy value, polarity index, and hydrophobic moment analysis (16, 27). The proposed signal sequence is indicated by the hatched box above residues -1 to -26.

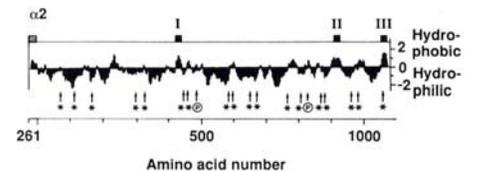


Fig. 3. RNA blot analysis of rabbit total RNA with (A) α_1 and (B) α_2 subunit cDNA probes. Total RNA was isolated from the tissues indicated, and 10 μ g of RNA per lane was electrophoresed in a 1% agarose-formaldehyde denaturing gel and transferred to nitrocellulose filters (28). The filters were hybridized with the respective cDNA probes and washed in 0.015M NaCl, 0.0015M sodium citrate, 0.1% SDS, pH 7.0, at 37°C for α_1 and 50°C for α_2 . The α_1 and α_2 cDNA probes, the Eco RI-Sac I fragment (nt 1006 to 5314) of pSkMCaCH α 1.7, and the cDNA insert (nt 133 to 3494) of λ SkMCaCH α 2.3 were labeled by random priming. Autoradiography was for 7 days with an intensifying screen, except for the RNA from ileum and aorta hybridized with the α_2 cDNA probe, which was exposed to x-ray film for 14 days.

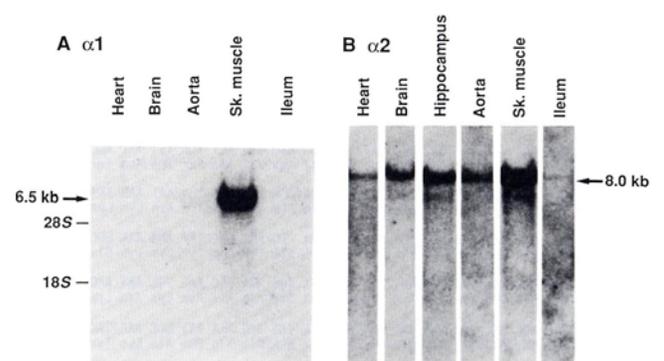
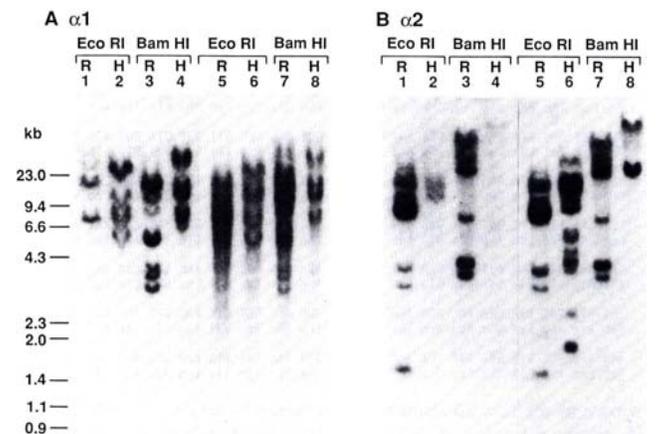


Fig. 4. DNA blot analysis of rabbit and human genomic DNA with (A) α_1 and (B) α_2 subunit cDNA probes. DNAs were digested with the indicated restriction enzymes and 15 μ g of digested DNA was electrophoresed in a 0.8% agarose gel and transferred to nitrocellulose. Blots were hybridized at 42°C in 0.25 mM EDTA, 0.05M NaH₂PO₄·H₂O, 0.9M NaCl, pH 7.0 [5 × saline sodium phosphate (SSPE)], 5 × Denhardt's solution, 50% deionized formamide, 0.2% SDS, and sonicated herring sperm DNA (200 μ g/ml) (29). (A) α_1 ; hybridization with pSkMCaCH α 1.7 (25) cDNA probe. (B) α_2 ; hybridization with cDNA insert of λ SkMCaCH α 2.3 (26) subcloned into pBI24 as probe. High stringency washing buffer consisted of 0.1 × SSPE, 0.1% SDS, 65°C (lanes 1 to 4). Low stringency washing buffer consisted of 1 × SSPE, 0.1% SDS, 50°C (lanes 5 to 8). R, rabbit genomic DNA; H, human genomic DNA.



brain. In contrast, an 8.0-kb transcript specific for the α_2 subunit was detected in total RNA from each of the tissues examined (Fig. 3B). In addition, a weakly hybridizing α_2 7.0-kb transcript was observed in skeletal muscle and the hippocampal region of the brain. The intensity of the α_2 hybridization signals also varied among the different tissues. This variability of the α_1 and α_2 hybridization signals may result from the absence or lower concentrations of the α_1 and α_2 transcripts in these tissues relative to skeletal muscle. Alternatively, the lower hybridization signals, or lack thereof, in certain tissues may be due to the presence of transcripts with varying degrees of sequence homology encoding α_1 and α_2 subunit-related subtypes.

To investigate the existence of gene sequences related to those encoding the skeletal muscle α_1 and α_2 subunits, rabbit and human genomic DNAs were digested with various restriction enzymes and DNA blots of these DNAs were hybridized with radiolabeled cDNA clones specific for the α_1 or the α_2 subunits (Fig. 4). Under conditions of high stringency, very few hybridizing bands were observed in rabbit genomic DNA with either the α_1 - or α_2 -specific probes (Fig. 4, A and B, lanes 1 and 3) indicating a low copy number, perhaps only a single copy each, of the α_1 and α_2 subunit genes. DNA blots of the same DNA preparations were also probed under conditions of low stringency with the same α_1 - and α_2 -specific probes (Fig. 4, A and B, lanes 5 and 7). Although more bands were observed with the α_1 cDNA probe than in the high stringency condition, no additional bands were observed with the α_2 cDNA probe. In addition, similar results were obtained with human genomic DNA (Figs. 4A and 4B, lanes 2, 4, 6, and 8), which suggests substantial conservation of the DNA sequences encoding the α_1 and α_2 subunits between rabbits and humans. These results, together with those of the RNA blot analyses, suggest that the α_1 subunit of the skeletal muscle DHP-sensitive Ca^{2+} channel may share homologous sequences with rabbit and human genes encoding distinct subtypes of Ca^{2+} channels. Additionally, these results confirm and extend those of recent peptide mapping and immunological studies that indicate that significant homology exists between the skeletal muscle α_2 subunit and a similar subunit associated with heart and brain L-type Ca^{2+} channels (17).

The sequence and structural homologies of the DHP receptor α_1 subunit with the α subunit of Na^+ channels (18) and a region of *Drosophila* K^+ channel proteins (19) support the conclusion that the α_1 subunit is part of a voltage-dependent Ca^{2+} channel.

Biochemical and ultrastructural evidence demonstrates that the α_2 subunit interacts with the α_1 subunit, and perhaps also with the β and γ subunits, with equimolar stoichiometry as a complex localized in the T tubules (3, 5-7). The apparent inability of mRNA derived from an α_1 subunit cDNA to induce the expression of voltage-dependent Ca^{2+} channel activity in *Xenopus* oocytes (20) suggests that, in contrast to Na^+ (18) and K^+ (19) channels, the skeletal muscle DHP-sensitive Ca^{2+} channel may function only as a multiple subunit complex. Ion transport proteins and ligand-gated ion channels, such as Na^+, K^+ -ATPase (21) and the nicotinic acetylcholine receptors (22), require multiple, distinct subunits for functions. For example, the α_2 subunit of the DHP-sensitive Ca^{2+} channel may be analogous to the β subunit of the Na^+, K^+ -ATPase, an extensively glycosylated protein with a single putative membrane spanning region, which has been proposed to be required for the insertion of the α subunit into the membrane (21).

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- Fragments of cDNA clones were subcloned into either pIBI24/25 (IBI, New Haven, CT) or M13 mp18/19. A sequential series of overlapping clones was generated prior to DNA sequencing [R. M. K. Dale, B. A. McClure, J. P. Houchins, *Plasmid* **13**, 31 (1985)]. The cDNA fragments were sequenced by the dideoxy chain-termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. The positions of nucleotide differences and the sequence determined are as follows: 2001 (C), 2049 (T), 2081 (G), 2556 (A), 2631 (C), 2835 (C), 2874 (T), 3222 (G), 3450 (A), 3462 (T), 3486 (G), 3498 (T), 3720 (T), 4212 (C), 4449 (C), 4515 (A), 4803 (C), 4854 (A), 4950 (C), 5190 (A), 5238 (A), 5423 (T), 5444 (T), 5504 (A), 5514 (C), 5631-5632 (CA insertion), 5655 (G), 5739 (C deletion), and 5855-5858 (CTGG deletion). All nucleotide differences resulting in amino acid differences were confirmed by sequencing in both directions. In two cases, clones $\lambda\text{SkMCA-CH } \alpha 1.4$ and $\text{pSkMCA-CH } \alpha 1.6$, significant alterations were found. The sequence of $\lambda\text{SkMCA-CH } \alpha 1.4$ diverges from the α_1 primary sequence at nt 3415, followed by a translational stop four co-

- dons farther in the carboxyl-terminal direction. The sequence of $\text{pSkMCA-CH } \alpha 1.6$ contains an in-frame 57-nt deletion from nt 3610 to 3666.
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- $\lambda\text{SkMCA-CH } \alpha 1.3$ nt 2847-3300, numbering according to (19)] was used to rescreen the λgt11 library and 8 of 1×10^6 clones were positive. Clone $\lambda\text{SkMCA-CH } \alpha 1.4$ (1700 nt) extended the farthest 5' to nt 2237. A fragment of this clone was used to screen 1×10^6 transformants of a rabbit back skeletal muscle cDNA library (constructed according to the method of H. Okayama and P. Berg). After an additional rescreening, the largest clone isolated, of eight positives, was $\text{pSkMCA-CH } \alpha 1.7$ (5.3 kb) extending 5' to nt ~450. Upon rescreening the λgt11 library, one clone, $\lambda\text{SkMCA-CH } \alpha 1.8$ (1.55 kb), was isolated that extended 78 nt 5' of the start codon.
- The nucleotide sequence shown was determined from clones $\lambda\text{SkMCA-CH } \alpha 2.15$ (nt -380-1123) and $\lambda\text{SkMCA-CH } \alpha 2.3$ (nt 133-3494). Five nucleotide differences among individual clones were observed, resulting in three amino acid changes. The nucleotides reported are from done $\text{SkMCA-CH } \alpha 2.3$. Differences occur in the sequence at positions 169, A(SkMCA-CH $\alpha 2.15$); 347 and 348, AA(SkMCA-CH $\alpha 2.2$); 984, T(SkMCA-CH $\alpha 2.4$); and a deletion of nts 1858-1860 (SkMCA-CH $\alpha 2.2$ and SkMCA-CH $\alpha 2.4$). The resulting amino acid changes are Asn⁹¹, Lys⁹⁰, and a deletion of Ser⁹⁴.
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