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Molecular cloning and nucleotide sequences of the complementary DNAs to chicken skeletal muscle myosin two alkali light chain mRNAs

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ABSTRACT

We report here the molecular cloning and sequence analysis of DNAs complementary to mRNAs for myosin alkali light chain of chicken embryo and adult leg skeletal muscle. pSMA2-1 contained an 818 base-pair insert that includes the entire coding region and 5' and 3' untranslated regions of A2 mRNA. pSMA1-1 contained a 848 base-pair insert that included the 3' untranslated region and almost all of the coding region except for the N-terminal 13 amino acid residues of the A1 light chain. The 741 nucleotide sequences of A1 and A2 mRNAs corresponding to C-terminal 141 amino acid residues and 3' untranslated regions were identical. The 5' terminal nucleotide sequences corresponding to N-terminal 35 amino acid residues of A1 chain were quite different from the sequences corresponding to N-terminal 8 amino acid residues and of the 5' untranslated region of A2 mRNA. These findings are discussed in relation to the structures of the genes for A1 and A2 mRNA.

INTRODUCTION

Chick embryo muscle cells have been extensively used as a model system for the study of the control of gene expression during cell differentiation.

Myosin is a major protein component in the contractile apparatus in the cell, consisting of two large subunits of 200 k daltons each and three to four smaller ones ranging from 15 to 30 k daltons (1). The fusion of mononucleated myoblasts into multinucleated fibers is associated with the synthesis of myosin peptides and other specific proteins involved in muscle contraction (2-7). Furthermore, multiple isozymic forms of the myosin heavy and light chains have also been identified (8-15). To study myosin gene expression, studies of DNA cloning of these proteins have been reported for chick embryonic cardiac myosin light chain (16), rat skeletal myosin heavy and light chains (17-19) and chick skeletal myosin heavy chain (20).

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Although, the primary structures of A_1 and A_2 light chains and DTNB light chains of rabbit skeletal muscle myosin have been known for some time (21), those of chicken skeletal muscle myosin were reported relatively recently (22, 23). As for chicken myosin light chains, the primary structures of heart and gizzard muscle myosin light chains have also been determined (23).

From a comparison of the primary structures of A_1 and A_2 light chains of chicken and rabbit skeletal muscle, Matsuda et al. (22) discussed a possible structure for these genes.

Here we describe the construction of a bacterial plasmid containing sequences of mRNAs for chicken skeletal muscle myosin light chains A_1 and A_2, and their nucleotide sequences. It was found that, while the 5' terminal region of the A_1 and A_2 mRNA is quite different, the 3' side of the mRNA including most of the coding region is identical.

MATERIALS AND METHODS

Preparation of mRNA

The microsome fraction was prepared from the leg skeletal muscles of 15 day chick embryos as described by Kennedy and Heywood (24) and poly(A)$^+$ mRNA was prepared as described previously (25).

For the preparation of poly(A)$^+$ RNA from 45 day chicken leg skeletal muscle, the method described by Duguid et al. (26) was used. Briefly, leg skeletal muscle was homogenized with 10 volumes of a buffer (0.1 M Tris-HCl pH 9.0, 13 mM DTT, 1% SDS) and 10 volumes of phenol-chloroform-isoamylalcohol (50:50:1) by a Waring blendor for 30 sec. After 5 times extraction, described above with the phenol-chloroform solution, a quarter volume of 10 M LiCl was added to the aqueous fraction containing total nucleic acid and the fraction was kept in ice for 1 h. Precipitated RNA was purified by two-cycles of oligo(dT) cellulose column chromatography.

Poly(A)$^+$ mRNA was then sedimented at 198,000 X g for 15 h on a 5-20% linear sucrose density gradient containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS in a Beckman SW 40 Rotor. Fractions (0.4 ml) were collected and assayed for translation of myosin alkali light chain with a nuclease-treated rabbit reticulocyte

lysate cell-free system (27). The translation products were analysed on 15% SDS acrylamide slab gel electrophoresis (34) followed by fluorography. Fractions containing mRNAs for myosin light chains were pooled and sedimented on a second sucrose gradient. As thus purified these are referred to as the enriched skeletal myosin light chain mRNA. For colony-filter hybridization, the enriched myosin light chain mRNA was further purified by 98% formamide acrylamide gel electrophoresis (32) and is referred to as the A_1 myosin light chain mRNA fraction (manuscript in preparation).

Preparation of double-stranded cDNA, insertion into pBR322, and transformation of χ1776

Complementary DNA molecule (cDNA) was synthesized from the enriched myosin light chain mRNA by using avian myeloblastosis virus reverse transcriptase (generously supplied by Dr. J.W. Beard, Life Sciences, St. Petersburg, FL), in a 400 µl reaction mixture containing 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 2.4 µg oligo$(dT)_{10}$ (Collaborative Research), 500 µM each dCTP, dGTP, dTTP, dATP (dCTP: ^{32}P radioactivity at the specific activity of 2.95 X 10^5 cpm/nmol) and 20 µg the enriched myosin light chain mRNA. The second strand of cDNA was synthesized by using a Escherichia coli DNA polymerase I (Klenow fragment, Bethesda research laboratories). After S1 nuclease treatment, the double-stranded cDNA was tailed with oligo$(dC)_{20-30}$ at the 3'-OH terminus by using calf thymus terminal transferase in the presence of $CoCl_2$ (28). The vector pBR322 was cleaved with restriction endonuclease PstI and was tailed with oligo$(dG)_{20-30}$ as described above. Oligo(dC)-tailed double-stranded cDNA was anealed with oligo(dG)-tailed plasmid DNA and then used to transform the EK_2 E. coli strain χ1776.

Bacterial colonies containing the recombinant plasmid were screened as described by Grunstein and Hogness (29) with highly labelled cDNA that was synthesized from the A_1 myosin light chain mRNA.

Identification of the plasmid by the positive hybridization translation method

Five µg of plasmid DNA was cleaved by restriction endonuclease Eco RI and coupled to DBM-paper after heat denaturation

according to the procedure of Hoeijmakers et al. (30).

Total poly(A)$^+$ RNA which was prepared from 45 day chicken skeletal muscle was hybridized to the coupled DNA and unbound RNA was washed off as described Hoeijmakeres et al. (30). The hybridized RNA was eluted from DBM-paper by heating for 1 min at 100°C in 200 µl 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.1% SDS and precipitated with 5 µg of carrier rat liver tRNA. The eluted RNA was assayed for the synthesis of myosin alkali light chain A_1 and A_2 by the reticulocyte lysate system (27). The products were analyzed by 15% SDS acrylamide slab gel electrophoresis (34) followed by fluorography.

Restriction endonuclease mapping of plasmid DNA

Conditions for restriction endonuclease cleavage of plasmid DNA were essentially as indicated by the suppliers. Fragments were electrophoresed on 1% agarose gels containing ethidium bromide and visualized by UV irradiation.

Restriction endonuclease Eco RI, BglII, PstI, AluI, HaeIII, KpnI, PvuII, HinfI and HpaII were purchased from Takara Shuzo Co. Ltd. and Sau3A from Bethesda Research Laboratories Inc.

Sequence determination of plasmid DNA

After restriction enzyme mapping of the cDNA clones, appropriate restriction fragments were labelled at their 5' ends with T_4 polynucleotide kinase (Takara Shuzo) and [γ-^{32}P]ATP (Amersham, 5,000 Ci/mmol) after removal of the 5' phosphate groups with bacterial alkaline phosphatase (Worthington). The 3' end of the PstI site was labelled with the T_4 DNA polymerase (Takara Shuzo) and [α-^{32}P]dCTP (Amersham, 2,500 Ci/mmol).

DNA fragments were separated on a thin polyacrylamide gel (8 and 20%) after chemical cleavage as described by Maxam and Gilbert (31).

RESULTS

Identification of recombinant plasmids containing cDNA sequence for mRNA of chick embryo myosin alkali light chain

Fifteen µg of poly(A)$^+$ RNA fraction enriched with the myosin alkali light chain A_1 mRNA that was prepared from 15 day chicken embryo leg skeletal muscle, was used to prepare the double stranded cDNA for the subsequent bacterial transformation.

About 5 X 10^5 tetracycline resistant χ1776 colonies were produced per 1 μg of double-stranded cDNA and 5 μg of oligo(dG) tailed pBR322. Five hundred transformed colonies were replica plated and screened, by colony-filter hybridization, with a ^{32}P labelled cDNA made from A_1 myosin light chain mRNA fraction which was purified by 98% formamide gel electrophoresis (32).

Recombinant plasmids were prepared from eight positive colonies and was assayed by positive hybridization-translation assay methods. Poly(A)$^+$ RNA prepared from 45 day chicken skeletal muscle was used for this assay, for the following reasons. This poly(A)$^+$ RNA fraction contained nearly equimolar amounts of mRNAs for A_1 and A_2 chains, while poly(A)$^+$ RNA prepared from 15 day chicken embryo skeletal muscle lacked mRNA for the A_2 chain (unpublished data). Furthermore, since the C-terminal 141 amino acid residues of A_1 and A_2 chains were identical (22), it was assumed that cDNA inserts of the myosin alkali light chain hybridized to both A_1 and A_2 mRNAs.

Of the colonies tested, one plasmid, pSMA-1, showed a fluorographic pattern of hybridization to both A_1 and A_2 mRNAs (data not shown), and was chosen for further characterization.

Partial restriction map of pSMA-1 and identification of chicken skeletal myosin alkali light chain sequence

The size of the inserted cDNA sequence was determined by digestion of pSMA-1 with PstI. A cleavage map of several restriction enzymes in pSMA-1 DNA was constructed by analyses of their single or double enzyme digests (Fig. 1). DNA of pSMA-1 was digested with BglII, and ^{32}P-end-labelled fragments were purified and sequenced as described above.

The nucleotide sequence determined, corresponded to the 170-190 amino acid residues of a chicken myosin alkali light chain and 3' untranslated regions. Plasmid pSMA-1 was thus confirmed as a clone for a chicken myosin alkali light chain.

Identification of recombinant plasmid containing chicken myosin alkali light chain A_1 or A_2 sequences

Poly(A)+ RNA was prepared from 45 day chick skeletal muscle and myosin alkali light chain mRNA was partially purified by sucrose density gradient centrifugation (data not shown) which separated A_1 and A_2 mRNA.

Poly(A)$^+$ RNA enriched with A_1 or A_2 chain was used to prepare the double stranded cDNA, which was used for subsequent bacterial transformation. One thousand transformed colonies were replica-plated and hybridized with ^{32}P labeled insert DNA of plasmid pSMA-1. The largest plasmid containing A_1 or A_2 sequence was selected from strongly hybridized colonies. Plasmid pSMA1-1 and plasmid pSMA2-1 was identified as a clone for A_1 chain and A_2 chain, respectively. Restriction maps of the insert DNAs of pSMA1-1 and pSMA2-1 are shown in Fig. 1.

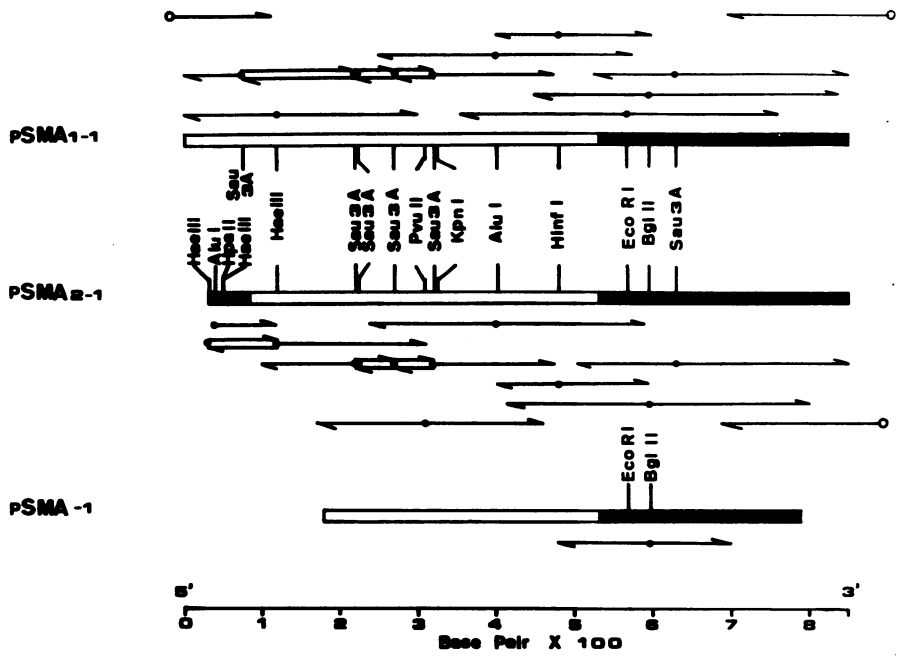

Fig. 1. Sequencing strategy and the restriction map of pSMA1-1, pSMA2-1 and pSMA-1. The open bar indicates the coding region of A1 or A2 mRNA, and the solid bar indicates the untranslated region of the corresponding myosin light chain mRNA. The restriction sites predicted by the DNA sequence were confirmed by digestion with restriction enzymes followed by sizing with 1% agarose gels. Restriction fragments were labeled at the sites of the solid circles and DNA sequencing proceeded in the direction of the arrow heads for the distance indicated by the length of the arrow. The 5'- and 3'-end regions of the cDNA were sequenced after labelling at the PstI site (open circle) by the T_4 DNA polymerase in the presence of α-^{32}P dCTP and 1 μM dCTP. The scale is indicated by the calibration in base pairs and shown at the bottom of the figure.

Sequence determination of pSMA1-1 and pSMA2-1

The nucleotide sequences of chicken skeletal muscle myosin A_1 and A_2 mRNAs were determined (Fig. 2). The strategy of

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                1
pSMA1-1         A CCA GCA CCC GCA CCC GCG CCA GCA CCT GCA CCA GCG CCC GCT AAA CCC
                  Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Lys Pro
pSMA2-1                                                   C CTC TCA GCT AAT CCC TCC
50                                                                          100
AAA GAA CCA GCC ATC GAC CTC AAG AGC ATC AAG ATC GAG TTC TTC AAG GAG CAG CAG GAT*
Lys Glu Pro Ala Ile Asp Leu Lys Ser Ile Lys Ile Glu Phe Ser Lys Glu Gln Gln Asp
GGC CCG TCC GCT CTA CTT TTC CAA CTC TCA ATC ATG TCC TTC TCA CCT GAC GAA ATC AAT
                                                Ser Phe Ser Pro Asp Glu Ile Asn

                                                            150
GAC TTC AAG GAG GCC TTC CTC CTC TTT GAC AGG ACT GGT GAT GCC AAG ATT ACC CTG AGC
GAC TTC AAG GAG GCC TTC CTC CTC TTT GAC AGG ACT GGT GAT GCC AAG ATT ACC CTG AGC
Asp Phe Lys Glu Ala Phe Leu Leu Phe Asp Arg Thr Gly Asp Ala Lys Ile Thr Leu Ser
                                        200
CAG GTC GGT GAC ATC GTT CGG GCA CTG GGA CAG AAC CCC ACA AAC GCT GAG ATC AAC AAG
CAG GTC GGT GAC ATC GTT CGG GCA CTG GGA CAG AAC CCC ACA AAC GCT GAG ATC AAC AAG
Gln Val Gly Asp Ile Val Arg Ala Leu Gly Gln Asn Pro Thr Asn Ala Glu Ile Asn Lys
                    250
ATC CTG GGC AAC CCC AGC AAA GAG GAA ATG AAT GCC AAG AAG ATC ACT TTT GAA GAG TTC
ATC CTG GGC AAC CCC AGC AAA GAG GAA ATG AAT GCC AAG AAG ATC ACT TTT GAA GAG TTC
Ile Leu Gly Asn Pro Ser Lys Glu Glu Met Asn Ala Lys Lys Ile Thr Phe Glu Glu Phe
            300
CTG CCC ATG CTG CAA GCA GCT GCT AAC AAC AAG GAT CAG GGT ACC TTT GAA GAC TTT GTT
CTG CCC ATG CTG CAA GCA GCT GCT AAC AAC AAG GAT CAG GGT ACC TTT GAA GAC TTT GTT
Leu Pro Met Leu Gln Ala Ala Ala Asn Asn Lys Asp Gln Gly Thr Phe Glu Asp Phe Val
350                                                                         400
GAA GGT CTG CGT GTT TTC GAC AAG GAA GGC AAC GGC ACA GTC ATG GGG GCT GAG CTC CGC
GAA GGT CTG CGT GTT TTC GAC AAG GAA GGC AAC GGC ACA GTC ATG GGG GCT GAG CTC CGC
GlU Gly Leu Arg Val Phe Asp Lys Glu Gly Asn Gly Thr Val Met Gly Ala Glu Leu Arg
                                                    450
CAT GTA CTG GCT ACA CTG GGC GAG AAG ATG ACA GAA GAG GAA GTA GAA GAA CTG ATG AAA
CAT GTA CTG GCT ACA CTG GGC GAG AAG ATG ACA GAA GAG GAA GTA GAA GAA CTG ATC AAA
His Val Leu Ala Thr Leu Gly Glu Lys Met Thr Glu Glu Glu Val Glu Glu Leu Met Lys
                                        500
GGT CAG GAA GAC TCC AAC GGC TGC ATC AAC TAC GAG GCA TTT GTA AAG CAC ATC ATG TCT
GGT CAG GAA GAC TCC AAC GGC TGC ATC AAC TAC GAG GCA TTT GTA AAG CAC ATC ATG TCT
Gly Gln Glu Asp Ser Asn Gly Cys Ile Asn Thr Glu Ala Phe Val Lys His Ile Met Ser
                    550
GTC TAA GAG GAC GTC CCC AGA TAA CAA ATG AAC AAG TTG AAT TCG GAA AGA CCA GTA GGC
GTC TAA GAG GAC GTC CCC AGA TAA CAA ATG AAC AAG TTG AAT TCG GAA AGA CCA GAT GGC
Val ∞
            600
ACC TAA GAT CTG CCT GAG ATG AAA ATT CTT GCT CTG GCA GAT CAT CAT CAA CCA TGG AAA
ACC TAA GAT CTG CCT GAG ATG AAA ATT CTT GCT CTG GCA GAT CAT CAT CAA CCA TGG AAA
650                                                                         700
CTG CGT GAC TGG ACA ATA TGC CTC CAA ACC CAC TAC TTT TCT TTT TAA CCG TGC ATC CAT
CTG CGT GAC TGG ACA ATA TGC CTC CAA ACC CAC TAC TTT TCT TTT TAA CCG TGC ATC CAT
                                                    750
GGA CAT TGC CTC TGG GTC ACT TCC AAA ACT GAA CTT TCA CGG GGG TTG TGG AAA CTG ACC
GGA CAT TGC CTC TGG GTC ACT TCC AAA ACT GAA CTT TCA CGG GGG TTG TGG AAA CTG ACC
                                        800
ACT GCC GCA CCA CCA CCC AAC ACC TCT GTG TTC CTG AGA TTG ACC TCC GAA AAT AAA GCA
ACT GCC GCA CCA CCA CCC AAC ACC TCT GAG TTC CTG AGA TTG ACC TCC GAA AAT AAA GCA
                        848
TCA GTA AAT CCC TGT TGT CAA AAA AAA AAA AAA AAA AAA
TCA GTA AAT CCC TGT TGT CAA AAA AAA AAA AAA AAA AAA
```

Fig. 2. Nucleotide sequences and predicted amino acid sequences of pSMA1-1 and pSMA2-1 cDNAs. The amino acid sequence was deduced from the sequence of cDNA using the genetic code. ∞, the ochre terminater. *, shows the first nucleotide of the identical 741 nucleotides between A_1 and A_2 cDNAs.

sequencing and the restriction map of three cDNAs are shown in Fig. 1. The sequence determination was performed on both strands of the cDNA except for the 3'-terminal 219 base pairs. The inserted sequence of pSMA1-1 corresponded to amino acid residues 15 to 190 of the A_1 chain determined by Matsuda et al. (22) and to the 3'-untranslated region extending to the poly(A) segment. The codon for the valine residue at the carboxy terminus of A_1 chain is followed by the translational termination codon TAA. Thus the 3'-untranslated region of A_1 mRNA is 315 nucleotides long (excluding the poly(A) tract), a putative poly(A) signal AATAAA is present 22 bases away from poly(A) sequence.

The insert of pSMA2-1 covered the coding sequence for amino acid residues 1 to 149 of the A_2 chain (22) and the untranslated sequence for the 5'- and 3'-end regions. The translational initiator codon ATG is followed by the serine codon of the amino terminus of the A_2 chain.

These sequences disagree at two positions with a previous report (22) determined by amino acid sequencing techniques. At positions 6 and 8 of the A_2 chain, we identified Glu and Asn, while Matsuda et al. reported Gln and Asp, respectively.

Comparisons of nucleotide sequences of pSMA1-1 and pSMA2-1

The sequences of pSMA1-1 and pSMA2-1 were aligned to examine their homology. As shown in Fig. 3, the 741 nucleotides from residues 108 to 848 of pSMA1-1 and those of 78 to 818 of pSMA2-1 agreed completely. This contains not only the sequences of the coding region corresponding to the carboxy-terminal 141 amino acid residues of the A_1 and A_2 chains, but also all the 3'-untranslated regions of A_1 and A_2. On the other hand, the 5'-terminal 108 nucleotides of pSMA1-1 and 78 nucleotides of pSMA2-1 were quite different, showing no homology between them.

DISCUSSION

The primary structures of skeletal muscle myosin A_1 and A_2 chains of the rabbit (21) and those of the chicken (22) have been determined already. As shown in Fig. 3(A) the C-terminal 141 amino acid residues of chicken A_1 and chicken A_2 are identical. Those of rabbit A_1 are identical with those of rabbit A_2. Furthermore, not only the positions of amino acid substitution but also the kinds of the substituted amino acids between chicken

A_1 and rabbit A_1 are identical with those between chicken A_2 and rabbit A_2. On the other hand, the sequences of N-terminal 50 amino acid residues are quite different between chicken A_1 and rabbit A_1. The N-terminal 8 residues of chicken A_2 are also different from the corresponding residues of rabbit A_2.

Fig. 2 shows the comparison of the nucleotide sequences of mRNAs of chicken skeletal muscle light chain A_1 and A_2, which are

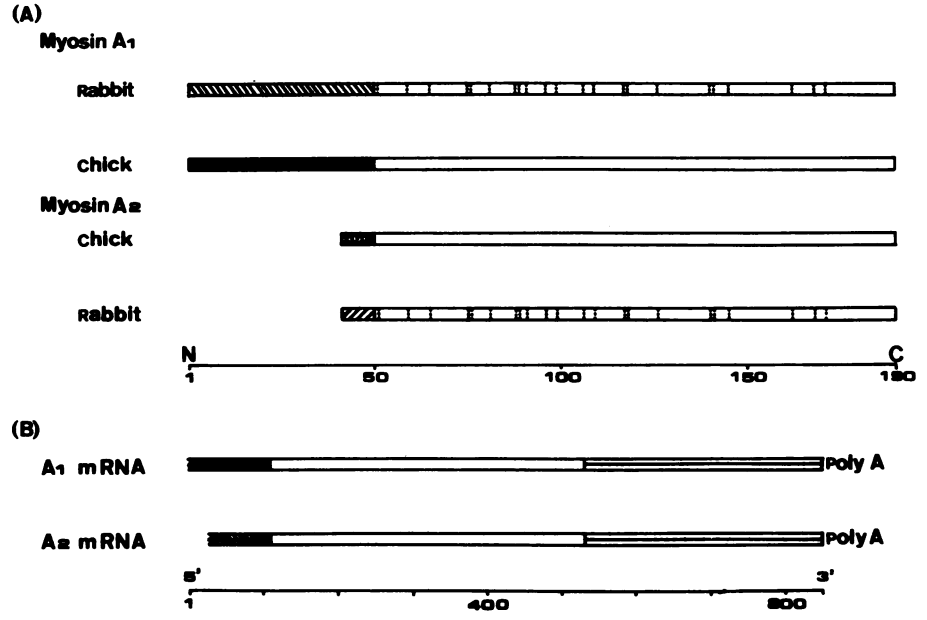

Fig. 3. Schematic diagrams of the primary structures of A1 and A2 peptides of chicken and rabbit skeletal muscle myosin (A) and nucleotide sequences of mRNAs for chicken skeletal muscle myosin A1 and A2 (B). A. the open bars indicate the common region of A1 and A2 peptides of chicken and rabbit skeletal muscle. The dashed lines in the open bars showing rabbit A1 and A2 peptides show the amino acid substitutions between chicken and rabbit skeletal muscle myosin alkali light chains. The solid and shaded bars show the A1 and A2 specific regions, respectively. The amino acid substitutions in A1 and A2 specific regions between chicken and rabbit skeletal myosin are not shown. B. The open bars and the open bars with center line indicate the identical 741 nucleotides of A1 and A2 mRNAs corresponding to common region of A1 and A2 peptides (open bars) and 3'-untranslated regions (with center line), respectively. The solid and shaded bars show the A1 and A2 specific nucleotide sequences of mRNAs, respectively. Dotted area indicates the A2 specific 5'-terminal amino acid coding sequence and shaded area represents the leader sequence.

schematically described in Fig. 3(B). The 3'-terminal nucleotide sequences of chicken A_1 and A_2 are identical, which contain not only the corresponding region to C-terminal 141 amino acid residues but also contain the following 3'-untranslated region. On the other hand, 5'-terminal nucleotide sequences of A_1 mRNA corresponding to the N-terminal 36 amino acid residues of A_1 light chain are different from those of the A_2 mRNA corresponding to the N-terminal 8 amino acid residues of A_2 peptide and the 5'-untranslated region.

What is the relationship between the genes for the A_1 and A_2 proteins? There are two possibilities. One is that the A_1 and A_2 peptides are encoded by two distinct genes. The other is that while the 5'-terminal regions of the A_1 and A_2 mRNAs are encoded in two separate DNA segments, their 3'-terminal 741 nucleotides are encoded in a common DNA segment on one gene. The total identity of the relatively long, 3'-terminal sequences of the mRNA, suggests the latter possibility. Two mechanisms have been reported for the phenomenon that genes for two or many kinds of proteins contain one DNA segment in common. One is the gene recombination that takes place on immunoglobulin genes (33, 34), and the other is RNA-RNA splicing that has been shown in the expression of either secreted and membrane types of immunoglobulin μ chain and δ chain, (35, 36) or α-amylase mRNAs in two different tissues; the salivary gland and the liver (37). Experiments to distinguish between these two possibilities in the case of chicken myosin light chain A_1 and A_2 are now in progress.

It is known that chicken myosin A_2 chain is not synthesized in the embryonic muscle. It will be interesting to know the mechanism by which the pattern of gene expression changes from only the A_1 peptide to the A_1 plus A_2 peptides during the course of skeletal muscle differentiation.

For the case of the myosin alkali light chains, heart and gizzard muscles are composed of one alkali light chain homologous to skeletal muscle A_1 and A_2 light chains, respectively (23). It is also of interest as to why heart and gizzard muscles express only one type of alkali light chain, while skeletal muscles express both types.

During the preparation of this manuscript, Robert et al. (38)

reported the cloning of cDNA for an alkali light chain of mouse skeletal muscle myosin and determined the partial nucleotide sequences of its 3'-untranslated region.

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