論文題目　骨格筋型速筋型ミオシンアルカリ
軽鎖遺伝子の発現機構の解析

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ABSTRACT

I characterized the upstream regulatory region of the chicken alkali light chain (MLC1f/MLC3f) gene as a muscle-specific enhancer (MLC1 enhancer). The essential sequence for ehnancing activity had two MyoD binding sites, each of which was indispensable for transcriptional activation. By using a gel mobility retardation assay, a muscle-specific nuclear factor(s) that binds specifically to this region was detected. The binding was inhibited by addition of muscle creatine kinase (MCK) enhancer sequence as a competitor. This suggests that a common or related factor(s) binds to the MLC1 enhancer and the MCK enhancer and regulates the expression of these genes.

Recent studies have shown that several myogenic factors including MyoD and myogenin directly bind to muscle specific regulatory elements and that they trans-activate the expression of a number of muscle specific genes, suggesting that they are involved in terminal muscle differentiation events. To elucidate whether these factors directly bind to the MLC1 enhancer, I expressed cDNAs for chicken myogenin, chicken MRF4, and CMD1 (chicken MyoD) in E. coli. The gel mobility retardation assay by using respective factors revealed that they bound to the MLC1 enhancer, but the mobility of the each retarded band was different from that with a muscle nuclear extract. On the other hand, when a liver nuclear extract was supplied to CMD1 or myogenin, a specific retarded band, the mobility of which was similar to that with a muscle nuclear extract, was detected. Furthermore, with MRF4, such a band was hardly

detected if at all. The binding experiments implicate that CMD1 or myogenin binds to the MLC1 enhancer by forming a complex with ubiquitous factor(s) such as E12/E47, as in the case of the binding of MyoD-E12 or MyoD-E47 to the MCK enhancer. Our observations of differential binding affinity of individual myogenic factors to the MLC1 enhancer suggest their distinct roles in gene regulation during muscle development.

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I. INTRODUCTION

Muscle cell differentiation in early development is thought that mesodermal multipotent precursor cells are determined to myoblasts, which consequently fuse with one another to terminally differentiate to make myotubes. Many muscle contractile proteins and muscle-specific enzymes are known to be transcriptionally activated coordinately at the time of terminal muscle differentiation. To understand the mechanism of transcriptional regulation of the muscle specific genes, a number of laboratories have identified specific DNA regulatory sequences that control the expression of several muscle specific protein genes, including those encoding α-actin (34), myosin light-chain 1/3 (MLC1f/MLC3f) (51, 15, 50), myosin light-chain 2 (6), myosin heavy chain (3), troponin I (TNI) (61, 43), troponin T (32), muscle creatine kinase (MCK) (24, 10, 23, 19) and acetylcholine recepter (AChR) α-subunit (47).

Nabeshima et al. have analyzed the myosin alkali light chain gene family to study the mechanism of transcriptional expression of these genes. In the following, I attempt a general overview of myosin structure and function.

Myosin is a major constituent protein in muscle cells, in which it is responsible for the muscle contraction through the sliding of myosin filaments and actin filaments over each other. Myosin molecule is composed of two heavy chains (MHCs) and four associated light chains (MLCs). MHC has a molecular weight of 200,000 and consists of a globular head region and α-helical rod-like tail. The head region has an actin binding ability and ATPase activity. MLCs, whose molecular weight are in the range of 15,000-30,000, are classified into two different functional classes; regulatory type (MLC2) and

alkali type (MLC1 and MLC3). In smooth and nonmucle cells, the regulatory MLC regulates the actin-myosin interaction by being phosphorylated. Site directed mutagenesis of a chicken skeletal MLC2 cDNA has identified specific functional domains in the MLC2 protein (46). Alkali MLCs dissociate from myosin molecules in an alkali solution. The function of alkali MLCs is poorly understood.

Alkali MLCs have multiple isoforms and are encoded by a multigene family. Of the chicken alkali MLCs, Nabeshima et al. have isolated the cDNAs for skeletal (MLC1f/MLC3f) (38), cardiac (42), embryonic (L23) (25), and smooth muscle MLCs (40). They have also isolated chromosomal genes of skeletal (41), cardiac (42), and L23 (39). Nabeshima et al. (41) have reported that MLC1f/MLC3f gene produces two kind of transcripts (MLC1 mRNA and MLC3 mRNA) by using two transcriptional start sites and by alternative splicing. The MLC1 mRNA is transcribed from the 1st exon, and the transcription is dramatically activated when myoblasts fuse with one another to form myotubes, and this stage is correspondent to 12- to 14-day-old embryo in ovo. The MLC3 mRNA is transcribed from the 2nd exon, and its transcription is activated at later stage than that of MLC1 activation. MLC isoforms are expressed in an orderly fashion under tissue-specific and developmental stage specific controls (see Fig. 1). MLC1, cardiac, and L23 genes are all expressed in skeletal muscles during early developmental stages of the chicken embryo. However in adult muscles, only the MLC1/MLC3 gene is still active in spite of the disappearance of other MLCs (45, 44, 25, 39). The molecular mechanism of expression of different MLC isoforms at different stages of muscle development is not well known.

Recently, several myogenic determination factors, MyoD (12, 30, 22, 7, 14), myogenin (59, 16, 7, 1), Myf-5 (8) and MRF4 (48, 33) have been identified in various species and each of them was found to convert C3H10T1/2 fibroblasts to myoblasts when their respective cDNAs are expressed under the control of a viral promoter. These proteins are structurally related, sharing a common basic region, that is required for sequence-specific DNA binding, and a helix-loop-helix (HLH) domain required for protein oligomerization with E12/E47 (35, 36, 9). It was first reported that MyoD could bind to the muscle creatine kinase (MCK) enhancer in vitro (28), and could trans-activate the MCK enhancer in vivo (13). Similar results have been reported between myogenin and the MCK enhancer (9) or the AChR α-subunit enhancer. These data suggest that myogenic factors act as trans-regulators by binding to the regulatory regions of terminally induced muscle protein genes.

This dissertation aims at the analysis of the molecular mechanisms of expression of MLC1 gene during development. Shirakata et al. (51) have shown the existence of a positive regulatory element in the region from -2096 to -1936 upstream of the MLC1f mRNA initiation site, which is activated in muscle cells. In this dissertation, the positive regulatory element of chicken MLC1f/MLC3f gene (51) was characterized, and was defined as a muscle-specific enhancer (MLC1 enhancer) (26). Deletion mutants of the MLC1 enhancer with homologous promoter region jointed to the chloramphenicol acetyltransferase (CAT) gene revealed that the essential sequence for enhancing activity had two putative MyoD binding sites tandemly repeated. The existence of these two sites were indispensable for the function as an enhancer. A gel mobility retardation assay revealed that a common or related muscle-

specific nuclear factor(s) bound to the MLC1 enhancer and the MCK enhancer.

Second, we expressed the cDNAs for several myogenic factors including CMD1 (30, 37), chicken MRF4 (37), and chicken myogenin (1) in E. coli to determine whether they bind to MLC1 enhancer by using the gel mobility retardation assay. Respective proteins specifically bound to the MLC1 enhancer, but the mobility of the each retarded band was distinctly different from that with the muscle nuclear extract. Then, they were mixed with a liver nuclear extract, for MyoD or myogenin was found to form heterocomplex with ubiquitous E12/E47 to get high affinity to MCK enhancer (36, 9). With CMD1 or myogenin, a new retarded band similar to that with the muscle extract was detected, but with MRF4, such a band was hardly detected if at all, implying that each myogenic factor plays different roles in controling muscle-specific gene expression during muscle differentiation.

II. MATERIALS AND METHODS

MATERIALS.

The materials used in this experiment were purchased from TAKARA, New England Biolabs, FUNAKOSHI, Amersham, GIBCO, and SEIKAGAKU KOGYO Co. LTD..

METHODS

Construction of the LC1-CAT fusion genes. To test the enhancing activity, the upstream regulatory region of the MLC1f/MLC3f gene was jointed with its promoter region, which was located from -299 to +64 of the transcriptional start site of the gene. The SalI site of pLC299 (51), which was located at 5' end of the inserted promoter sequence, was previously converted BglII site by linker ligation (pLC299B). A DNA fragment of the upstream regulatory region (-2096 to -1940) was subcloned into the polycloning site of pUC119, of which the SalI site was converted to BglII site (pLCBB). The regulatory region was cut out again by BglII and BamHI and was inserted into the upstream BglII or the downstream BamHI site of pLC299B in both orientation respectively. The plasmid, in which the regulatory region was inserted into the upstresm BglII site of pLC299B in correct orientation, was designated pLCCAT(-2096/-1940).

To make deletion constructs of the enhancer, the regulatory region was deleted from 5' end or from 3' end by Bal 31 nuclease (TAKARA) and were jointed with the promoter region of pLC299B in correct orientation (pLCCAT series). To make internal deletion

constructs of the enhancer, the fragment (-2047 to -1995) of the ELC1 was cut out with PstI and 5' and 3' fragments were ligated with one another, or 3' end of the 5' fragment of the ELC1 was deleted by Bal31 nuclease, and ligated with PstI linker, followed by ligation with 3' fragment. Then, the internally deleted fragments were inserted into pLC299B.

To make pLCCAT constructs of which the promoter regions are exchanged to the herpes simplex virus thymidine kinase (TK) promoter, and the MLC3 promoter region (-263 to +36), the MLC1 promoter region was cut out from pLCCAT(-2096/-1940) at BglII and HindIII site and exchanged to the respective promoter fragments, linked with BglII and HindIII linkers, in correct orientation.

Substitution mutations in the enhancer region were introduced to pLCBB according to the method of Kunkel (27) with oligonucleotides containing the mutated sequence synthesized with a nucleotide synthesizer (model 381A; Applied Biosystems, Inc.), and mutated fragments were inserted into pLC299B.

Cell culture, DNA transfections, and CAT assays. Primary cultured muscle cells or fibroblasts were prepared from the breast muscle or the skin tissue of 12-day-old chicken embryos. The minced tissues were digested breafly with trypsin (0.05%,w/v) at 37°C for 10 min and then pipetted several times. Isolated cells (2×10^6 per a 6-cm gelatin coated dish) were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% horse serum and 4% chick embryo extract. Transfection was carried out by using the DEAE dextran method (31), in which 15μg of plasmid DNA in 1.5 ml of DEAE dextran medium (80μg/ml) per a dish was added 12 h.

after plating for 2 h., and the cells were treated with 10% (v/v) DMSO in serum-free DMEM for 2 min.. The cells were harvested 48 hours after transfection, when almost all myoblasts fuse with one another to make myotubes, for CAT assay.

CAT activity was assayed as described previously (17). Cell extracts were prepared by freeze-thawing the harvested cells three times in 100 μl of 0.25 M Tris-HCl (pH.7.5), followed by centrifugation for 10 min. in an Eppendorf microfuge at 4°C. All enzyme reactions were performed for 1 h. as duplicate, independent transfections and normalized to total protein (20μg) in extracts.

Construction of bacterial expression vectors. Respective cDNAs for CMD1 (37), chicken myogenin (1), and chicken MRF 4 (37) were expressed in E.coli using T4 polymerase system (purchased from Novagen) according to the method of Studier et al. (53). The subcloned plasmids for CMD1 and myogenin cDNAs were partialy cut at NcoI site, at which translational start ATG sequence exists, and ligated with 10-mer BamHI linker. Each EcoRI site of 3' untranslated flanking region was converted to BglII site by linker ligation. Then, BamHI-BglII fragments were inserted to the BamHI site of the expression vector, pET-3a, named pET-CMD1 and pET-myogenin respectively. For MRF4 expression, both flanking EcoRI sites were converted to BamHI sites by 10-mer linker ligation, and the BamHI fragment was inserted to the BamHI site of pET-3c (pET-MRF4). The control plasmid, pET-1DMC, was made by inverted insertion of CMD1 fragment at the BamHI site of pET-3a. Expressed proteins contain 15 amino acid excess in CMD1 and in myogenin, and 21 excess in MRF4 from the amino terminus methionine. All constructs were confirmed to contain the fusion gene in the correct

reading frame by sequencing. Expressed proteins were identified by Western blot analysis (56) with monoclonal antibody (MG7C6) against myogenin which also reacts with CMD1 and MRF4. The concentration of the expressed proteins in the extracts was calibrated and normalized by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (29) and coomassie staining using bovine serum albumin (BSA) as a standard.

Preparation of nuclear extracts and **E. coli** lysates and a gel mobility retardation assay. Nuclear extracts were prepared by the method of Gorski et al. (18) from the breast muscles and the livers of 12- and 13-day-old chicken embryos. Protein concentrations in the extracts were measured by the method of Bradford (4). The E. coli lysates that contain CMD1, myogenin, and MRF4 proteins were prepared as follows: plysS transformed with various vectors was grown in 500ml of LB medium, at an OD_{600} of 0.4 units, 0.4 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cells were incubated at 37°C for 45 min, followed by centrifugation. The cell pellets were extracted with about 40 vol. of solution A (20mM Hepes [pH7.9], 1mM EDTA, 20% (v/v) glycerol, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 1mg/ml of leupeptin, 1mg/ml of pepstatin) with NaCl to final concentration of 20mM, to extract soluble proteins, and were centrifuged at 48,000 rpm. for 1 h.. Then the pellets were extracted with 4 ml of high ionic strength solution (solution A containing 1M NaCl) to extract respective proteins followed by ultracentrification at 76,000 rpm.. Supernatants were aliquoted and stored in liquid nitrogen. A gel mobility retardation assay was performed as follows. The sense and antisense oligonucleotides for the probe and the

competitors were synthesized, annealed and then end labeled (2×10^6 cpm/pmol) with [γ-^{32}P]ATP (ICN). The reaction mixture (17μl) for the gel mobility retardation assay contained 0.01 pmol of the labeled probe, 4 μg of nuclear extract or normalized E.coli lysates containing 25 ng of respective factors, and 4 μg of poly(dI-dC) as a nonspecific competitor and was adjusted to contain 10mM Tris-HCl (pH.7.5), 50mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA, 5% glycerol, 0.1% Triton X-100, and 0.5mM PMSF. The unlabeled probe or competitor at a 40-, 200-, 400-, 800-fold molar excess of the labeled probe was added. The reaction and electrophoresis were performed as described by Sing et al. (52).

Production of monoclonal antibodies. Monoclonal antibodies against CMD1 and myogenin were produced. The method to generate monoclonal antibodies in this experiment was described in ref. 21. Bacterically expressed CMD1 or myogenin, which were electrophoretically purified from SDS polyacrilamide gel electrophoresis gel and were mixed with Freund's complete adjuvant, were immunized to 10-week-old mice (BALB/c) intraperitonealy two times at about 10 days interval. Spleen cells were fused with myeloma cells (P3u1) with 50%(w/w) polyethylenglycol 4000 (MERCK) three days after last booster. Fused cells were selected in hypoxsanthine aminopterin thymidine (HAT)-RPMI 1640 mediun (Nissui Co.) supplemented with 15% FCS. Specific-antibody-secreting cells were identified by enzyme-linked immunosorbent assay (ELISA) or by immunoblotting with cultured supernatant of hybridomas. Antibody-producing cells were cloned by limiting dilution. Sub-typing of each positive antibody was

carried out by the sandwitch ELISA using subtype-specific antibodies (21).

III. RESULT

The upstream regulatory region functioned as a muscle-specific enhancer. Shirakata et al. (51) reported that a positive regulatory element exists in the region from -2096 to -1936 of the transcriptional start site of the chicken MLC1f/MLC3f gene, that is necessary for transcription induced during the differentiation of muscles. In an attempt to characterize the upstream regulatory region, we inserted the region into the upstream or the downstream of the MLC1 promoter region (-299 to +64) linked to the CAT gene in the pLC299B, to examine whether the upstream region works as an enhancer. Although a fusion gene pLC299B has TATA like sequence and CAAT box, and the MLC box (57), the transcription from the MLC1 promoter is not induced during muscle differentiation (51). When the upstream regulatory region was introduced into the upstream of the MLC1 promoter region, the CAT activity expressed in chicken primary myotube was dramatically enhanced in 12-fold to pLC299B (Fig. 2). When the regulatory region was introduced at upstream or downstream of the promoter in either orientation, transcription of the CAT gene was activated during muscle differentiation. The most enhancement (62-fold) was observed when the upstream regulatory region was located upstream in reverse orientation. These results clearly indicate that it functions as an enhancer in differentiated muscle cells. The upstream regulatory region was named ELC1 (enhancer of LC1).

In order to define the ELC1 as a muscle-specific enhancer, we exchanged the MLC1 promoter region to the herpes simplex virus TK promoter, or the MLC3 promoter region (-263 to +36). Introduction of the resultant plasmids exhibited the enhancing

activity in myoblasts, but not in fibroblasts (Fig. 3), indicating that ELC1 is a muscle-specific enhaner.

Identification of core sequences of ELC1 by 5' and 3' deletion analysis. To identify core sequences of ELC1, the effect of the deletion from 5' or 3' end of the ELC1 on the transcription of the reporter genes was examined. The CAT activities of 5' or 3' deletion mutants revealed that the enhancing activity reduced between the region from -1992 to -1974 (5' deletion) (Fig. 4) or from -1961 to -1970 (3' deletion) (Fig. 5). We named this region (-2005 to -1936) P region (proximal region). Since, a muscle specific nuclear factor was found to bind to 5' region (-2093 to -2040) of ELC1 (Shirakata, personal communication), we also constructed internal deletion mutants of ELC1 (Fig. 6). The CAT activity of deletion construct (Δ -2047 to -1995) showed about 2-fold to that of pLCCAT(-2096/-1936). The CAT activity of internal deletion constructs dramatically reduced between the region from -2068 to -2078, suggesting the existance of another responsible element, that enhances the activity of P region cooperatively. We don't examine this region in detail yet. Around the P region, a similar sequence was found in the enhancer of muscle creatine kinase (MCK) gene (10), containing two MEF1/MyoD binding sites. We found also two MEF1/MyoD-like sequences tandemly repeated around the the essential region of ELC1 (P1, P2 from 5' end respectively) (Fig. 7, Fig. 8, Fig. 9). Similar structure was also reported in the enhancer region of the chicken acetylcholine receptor α-subunit (AChR) gene (47), and each of them was found to be essential for sufficient enhancing activity.

Mutation analysis of ELC1. To ensure weather the existence of two MEF1/MyoD sites tandemly repeated may be essential for sufficient enhancing actitity, we constructed CAT fusion genes that was introduced mutations into each of the sites (Fig. 10). Mutations in either site of P1 or P2 (mutated sequences are listed in Fig. 9) dramatically reduced the activity of ELC1, indicating that both of the MEF1/MyoD binding sites are necessary for the enhancing activity of ELC1. The result suggests that the identical or related factor(s) binds to the homologous sequences and regulate the coordinate expression of muscle specific genes, involving MLC1, MCK, and AChR.

Nuclear factor(s) of skeletal muscle cells binds to P1 and P2 region of ELC1 **in vitro**. In an attempt to detect nuclear factors that specifically bind to the P1 or the P2 region of ELC1, we performed gel mobility retardation assays with a muscle nuclear extract or with a liver nuclear extract as control. Sequences of the probes used in these assays were listed in Fig. 9. When a synthetic oligonucleotide from the region of -2005 to -1953 including both the sequences of P1 and P2 was used as a probe (this region was confirmed to have enhancing activity), a muscle-specific retarded band was formed (Fig. 11). A muscle-specific retarded band was detected also with a probe of P2, but not with a mutant oligonuclotide of P2. The result shows that the sequence specificity of the binding of the P2 to the factor(s) coincides the sequence specificity of the enhancing activity of the ELC1. When P1 was used as a probe (Fig. 12), such a muscle-specific retarded band was not detected, but the specifically retarded band was thought to be overelapped with that of the liver nuclear extract. This was

confirmed by the competition assay described below. Then in order to know whether the P1, P2, and the MCK enhancer region share common factors, we performed gel retardation assays in the existence of exess amount of unlabeled oligonucleotides for P1, P2 and MCK enhancer oligonucleotides as competitors listed in Fig. 9. When we used P2 as a probe (Fig. 13a), the intensity of the retarded band was reduced by addition of exess amount of P1, P2 and MCK enhancer oligonucleotides, but not reduced by mutant oligonucleotides. But the level of competition was distinctly different among respective competitors. For example, when we added 200 molar excess competitors of P1, P2, and MCK to the probe, the intensity of the retarded bands were reduced to 65%, 30%, 5% to the control, respectively. Next, when we used the MCK enhancer as a probe (Fig. 13b), similar results of the competition were obtained, where 200 molar excess competitors reduced the intensity to 70%, 35%, and 15%, respectively. These results indicate that common or related factor(s) binds to the respective regions. We obtained another interesting observation: the retarded P2 band showed a secondary retarded band by addition of full length ELC1 (-2096 to -1936) as a competitor (Fig. 13a).

Production of monoclonal antibodies against myogenin and MyoD.
We established several monoclonal antibodies against myogenin and MyoD. By using immunoblot analysis, reactivity to the three myogenic factors was determined (Fig. 15). MG5C1, MG4H2, and MG6B4 reacted specifically to myogenin. MG7C6 reacted to myogenin, CMD1, and also MRF4. CD6B6 reacted specificically to CMD1. Subclass and cross-reacivity of each clone was determined.

Myogenic factors expressed in **E.coli** exhibit different binding specificities to P2 region of the MLC1 enhancer. We expressed respective cDNAs using bacterial expression vectors (Fig. 14) to examine whether the binding specificity to the P2 region was exhibited by three myogenic factors. Respective factors in the lysates were identified and normalized by SDS polyacrylamide gel electrophoresis and immunoblot with monoclonal antibody (MG7C6) against myogenin, which also reacts with CMD1, and MRF4. (Fig. 16). The gel retardation assays were performed with respective lysates containing about 25 ng of factor per reaction mixture (Fig. 17). Using either P2 or MCK enhancer as a probe, we could detect a sequence specific retarded band with all three lysates. But with P2 as a probe, the intensity of each retarded bands was much weaker than those with MCK enhancer. And the mobitily of the respective retarded bands were distinctly different from that with the muscle nuclear extract, which is consistent with the results reported previously (36, 9). It is reported that MyoD and myogenin associate with ubiquitous factors, E12/E47, which were identified as immunogloblin κ light-chain enhancer binging proteins, to make heterocomplexes to obtain higher affinity to MCK enhancer than MyoD or myogenin alone (36, 9). So the binding is considered to occur physiologically as a heterocomplex. So we mixed a liver nuclear extract with respective lysates to make hetero-complex with E12/E47, which was expected to be in liver nuclear extract. And the gel retardation assays were performed (Fig. 18). Using mixtures instead of the each lysate alone, an additional retarded band appeared when used CMD1 or myogenin lysate with either P2 or the MCK enhancer as a probe, and the mobility of the retarded bands was almost the same as that with the muscle nuclear extract.

In contrast, when we used the MRF4 lysate, a retarded band corresponding to that with the muscle extract was hardly detected. In order to investigate the binding specificity of the band, competition assays were performed (Fig. 19). The intensity of retarded band of P2 with either CMD1 or myogenin lysate was competed out by addition of 1000 molar excess of P2 or MCK enhancer sequence. These results indicate that hetero-complex of CMD1 or myogenin can bind to either P2 or the MCK enhancer in vitro, but that MRF4 can hardly bind to P2, although it can bind to MCK enhancer as a hetero-complex.

IV. DISCUSSION

In this report, first we defined the upstream regulatory element as an enhancer. To characterize the enhancer, we used upstream region from -299 to +64 of the transcriptional start site as a promoter region. In this region, the MLC box, which is conserved among myosin alkali light-chain genes, exists. Uetsuki et al. (57) reported that the MLC box of L23 showed tissue specificity in muscle cells. To exclude the possibility that the muscle-specific enhancing activity is not due to the ELC1, but due to the specificity of the promoter region, we constructed CAT fusion plasmids of which the promoter region was exchanged to the herpes simplex virus TK promoter, or MLC3 promoter region (-263/+36), which does not contain the MLC boxes to introduce them into chicken myoblasts. Using these promoters, ELC1 worked as an enhancer. We concluded that ELC1 is a muscle-specific enhancer. The isoform transition of the MLCs, including skeletal (MLC1/MLC3), cardiac (42), and embryonic (L23) MLCs, in developmental stages is reported (11, 44), in which these MLC genes are all expressed in skeletal muscles in early developmental stages, however in adult tissues, only the MLC1/MLC3 gene is active in contrast to the disappearance of other MLCs. We can now present a model of transition of the MLCs in skeletal muscles (Fig. 20). The CAT plasmids carrying the MLC boxes of the L23 (57) and the cardiac LC (Uetsuki et al., personal communication) gene showed relatively high activity in myoblasts, suggesting that expression of L23 and cardiac LC genes in embryonic skeletal muscle cells is due to the presence of the MLC boxes. On the other hand, the LC1 promoter-CAT fusion gene, pLC299B, did not show the transcriptional

activation, and the MLC box of the MLC1 gene also did not show the activation (Uetsuki et al., personal communication), suggesting that expression of LC1 gene in embryonic skeletal muscles is due to the existence of LC1 enhancer in the presence of MLC box as an upstream promoter. Interaction of ELC1 between the MLC1 box was not examined yet. Inactivation of L23 gene in adult muscle is inferred to be due to the existence of a negative regulatory element upstream of L23 gene (57). The existence of a negative regulatory region was suggested also in the cardiac LC gene (Uetsuki et al., personal communication). On the other hand, owing to the existence of the MLC1 enhancer, the MLC1 gene is still active in adult skeletal muscles. Donoghue et al. (15) and Seidel et al. (50) reported the downstream enhancer of rat and human MLC1f/MLC3f genes, respectively. The difference of the chicken upstream enhancer from those is that the former is not exchangeable to SV40 promoter (51), but exchangeable to LC3 promoter. But the latters are exchangeable to SV40 promoter, and human downstream enhancer dose not work with LC3 promoter (50). We have not screened the downstream enhancer yet, but it is possible to think that LC1 promoter and LC3 promoter use different enhancers or that the regulation of the expression of the LC1f/LC3f gene is different between mammal and Aves.

The core sequence of the ELC1 was found to have homology between MCK enhancer. Our data together with the data of MCK (28) and AChR (47) suggest that the existence of at least two MEF1/MyoD sites is required for adequate enhancer activity.

In vitro binding assay revealed that the ELC1 and the MCK enhancer bound to the common or related regulatory factor(s), but the difference of the capacity of competition between the two

enhancers exists indeed. For this reason we infer that the binding affinity between the two is different, which was confirmed by the gel mobility retardation assay, that P2 and the MCK enhancer showed different binding affinity using with factors expressed in E. coli. We obtained an interesting observation in the gel retardation assay; the retarded P2 band showed a secondary retarded band by addition of full length ELC1 as a competitor. This observation was specific for P2 as a probe, suggesting that one of the factors which binds to P2 region is different from what binds to the MCK enhancer or that one of the components of the complex that binds to P2 is different from what binds to the MCK enhancer.

Myogenic factors including CMD1, myogenin and MRF4 expressed in E. coli bound to P2 and MCK enhancer, although the binding affinity of the MCK enhancer seemed to be higher than P2 with any factors, which was consistent with the results using the muscle nuclear extract. MyoD and myogenin have been reported to associate with ubiquitous E12/E47 to get high affinity to MCK enhancer (36, 9). Myogenin was reported not to bind to MCK enhancer as a homo-complex in physiological condition (9), MyoD was not concluded to bind MCK enhancer as a homo-complex physiologically (36, 2). As to MLC1 enhancer, each factor does not seem to bind physiologically as a homo-complex, for about 25 ng per reaction mixture gave only slight retarded band in the gel retaration assay. So we added liver or cultured fibroblasts nuclear extract to the respective lysats in order to form hetero-complexes with ubiquitous factors like E12/E47. It is reported that Id (2), which is expected to exist in the liver extract, inhibits the formation of the complex between MyoD and E12/E47 by binding to E12/E47 with higher affinity. But we expected that larger amount of

expressed protein could compete out the formation of Id-E12/E47 complex. When we used P2 as a probe, a new retarded band similar to that with the muscle nuclear extract was detected with CMD1 or myogenin, but not or hardly detected with MRF4. In contrast, when used MCK enhancer as a probe, a new retarded band was detected with each factor. The different binding specificity between the muscle specific enhancers and myogenic factors was reported, where Myf-6 (human MRF4) showed lower affinity than other myogenic factors (9) to the human myosin light chain 1/3 downstream enhancer sequence.

We examined whether the respective factors could activate the MLC1 enhancer in vivo, by cotransfecting the eucaryotic expression vectors of respective cDNAs with a reporter plasmid, which contains upstrean regulatory region from +64 to -3381 of the MLC1 gene (51), showing that CMD1 and myogenin activated the transcription of this reporter plasmid, but that MRF4 did not activate the reporter plasmid (37). The binding specificity of respective factors to the MLC1 enhancer correlates with the results of trans-activation. With regard to CMD1 and myogenin, our observation is consistent with previously reported results on the MCK enhancer and the AChR enhancer (30, 13, 8, 5, 47, 60, 9,).

In contrast, concerning MRF4, it neither showed strong binding specificity to MLC1 enhancer nor it trans-activated reporter plasmid carrying MLC1 enhancer (37). Yutzey et al. (60) reported that MRF4 did not trans-activate reporter plasmids containing mouse MCK enhancer or chicken TNI enhancer like element. On the contrary, Braun et al. (5) reported that Myf-6 (human MRF4) trans-activated reporter plasmids containing human embryonic MLC promoter or human MLC1f/MLC3f downstream enhancer like element. These

complexity of trans-activation by MRF4 may reflect the functional specificity of MRF4. In our experiment, primary cultured chicken fibroblast, transfected with MRF4 expression vector, transiently expressed endogenous MLC1/MLC3 protein (37), suggesting that expression of the MLC1 gene requires other cis-elements besides the regulatory region used in the reporter plasmid. Alternatively, it is possible that endogenous CMD1 or myogenin which is activated by exogenous MRF4 activates, in turn, the MLC1/MLC3 gene. In order to analyze whether and how a myogenic factor activates its target genes, it is indispensable to analyze the cis-elements of the target gene in detail, and to analyze whether the myogenic factor acts to the restricted cis-element. In any case, we have shown that MRF4 activates the MLC1/MLC3 gene in a different manner from other two myogenic factors. Together with previous observations, the results presented here indicate that each myogenic factor may play different roles in activation of several muscle specific enhancers. But as a whole, myogenic factors, by regulating with one another to make a cascade or a network (52), may lead to a coordinate expression of muscle specific genes (see Fig. 21). Investigation of the differential roles of these myogenic factors will shed light on understanding the molecular mechanisms of the skeletal muscle differentiation.

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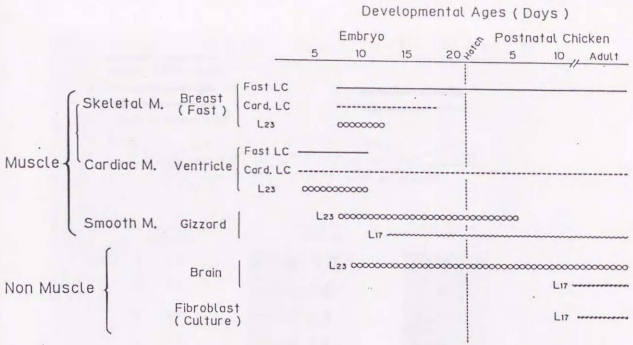


Fig. 1. **Schematic demonstration of MLC isoform transitions during embryonic and postnatal development of chicken.** Time course of the expression of fast skeletal type (Fast LC) (44, 45), cardiac type (Card. LC) (44, 45), embryonic type (L23) (25, 37), and smooth muscle/nonmuscle type (L17) (54) MLCs are shown.

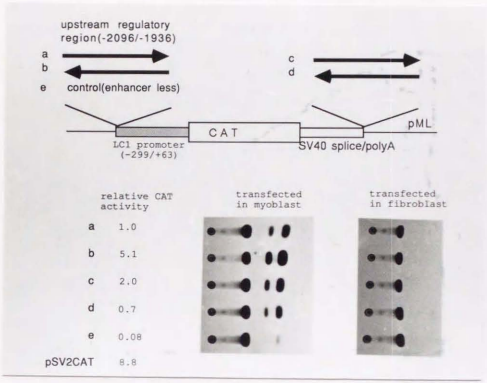


Fig. 2. **Enhancing activity of the upstream regulatory region of the chicken MLC1f/MLC3f gene.** Structure and expression of the MLC1f-CAT fusion genes. The upstream regulatory region (-2096 to -1936) was inserted into upstream or downstrem of the CAT gene linked to the LC1 promoter region (-299 to +64) in both orientation. The large arrows indicate inserted site of the upstream regulatory region and its orientation. The CAT activities of each plasmid in the primary cultured breast muscle cells were normalized to the activity of the plasmid, of which the regulatory region was inserted into upstream in correct orientation of the promoter region.

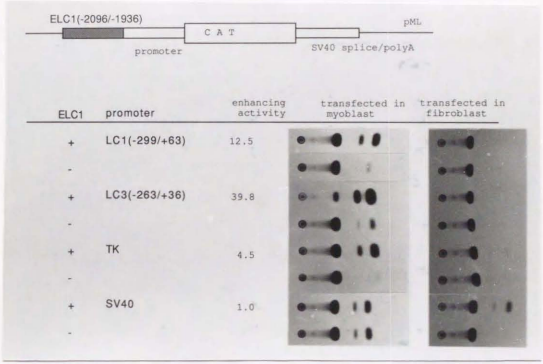


Fig. 3. **Enhancing activity of ELC1.** ELC1 was jointed to heterogenous promoters, including the LC3 promoter region (-263 to +36), the herpes simplex virus thymidine kinase (TK) promoter, and the SV40 early promoter, linked to the CAT gene. Enhancing activities indicate the ratio of the CAT activities of respective plasmids with ELC1 to that of the plasmids without ELC1 in muscle cells.

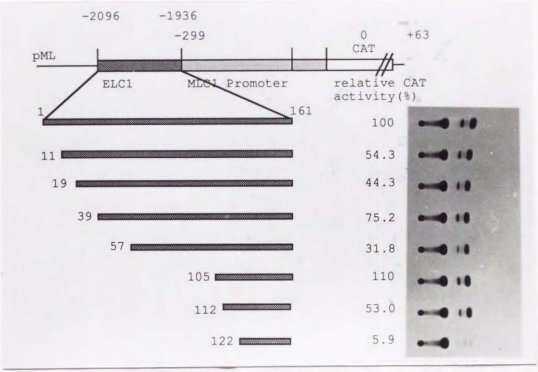


Fig. 4. **The CAT activities of the 5' deletion mutants of ELC1 in the primary cultured muscle cells.** A series of deletion mutants were obtained by digestion with Bal31 nuclease as described in Materials and Methods. Relative CAT activity of each plasmid is indicated. Mutant ELC1 deleted from 5' end are shown as stippled boxes.

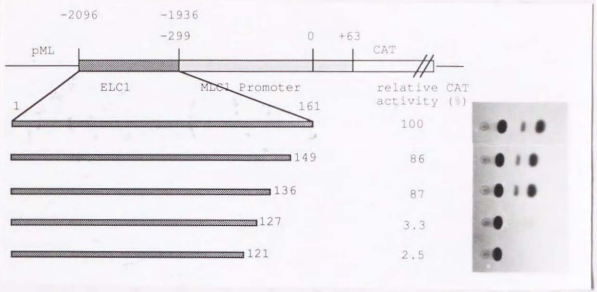


Fig. 5. **The CAT activities of the 3' deletion mutants of ELC1 in the primary cultured muscle cells.** Relative CAT activity of each plasmid is indicated. Mutant ELC1 deleted from 3' end are shown as stippled boxes.

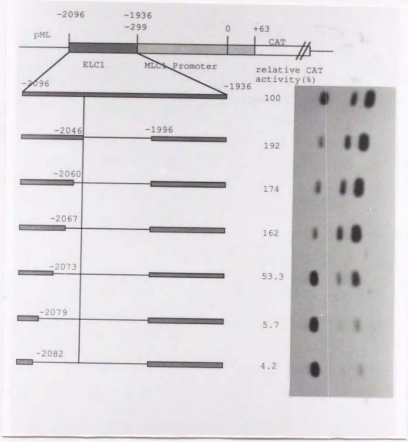


Fig.6. **The CAT activities of the internal deletion mutants of ELC1 in the primary cultured muscle cells.** Relative CAT activity of each plasmid is indicated. Holizontal lines indicate deleted sequence. Vertical line indicates PstI site.

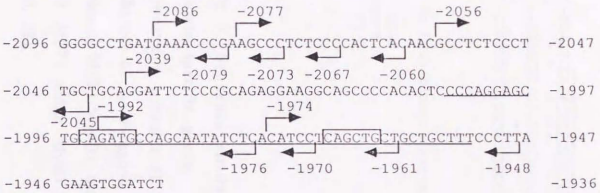


Fig. 7. **Nucleotide sequence of ELC1.** The end points of the various deletion mutants are indicated by arrows. Open boxes indicate the putative MyoD/MEF1 binding sites (10, 28). The underlined sequence is used as a probe for gel mobility retardation assay.

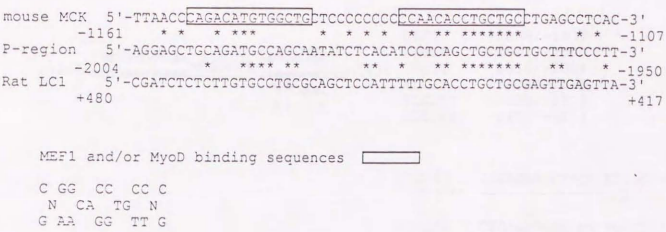
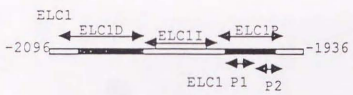


Fig. 8. **Sequence similarity of enhancer elements of various muscle specific genes.** Mouse MCK enhancer (10) and rat LC1 downstream enhancer (15) sequence are compared with the enhancer of the P-region. Stars indicate homologous sequence. Boxes indicate MEF1/MyoD1 binding sequence reported previously (10, 28). MEF1 and/or MyoD binding consensus sequences are shown (10, 28).



ELC1	-2096/-1936
ELC1D	-2093/-2040
ELC1I	-2039/-2006
ELC1P	-2005/-1953
ELC1P1	-2005/-1979
ELC1P2	-1978/-1953

ELC1P1	CCCAGGAGCTGCAGATGCCAGCAATAT
ELC1P1M	----------CA----------
ELC1P2	CTCACATCCTCAGCTGCTGCTGCTTT
ELC1P2M	----------GGTAAC-------
MCKW	CCCCCCAACACCTGCTGCCTGAGCC
MCKM	----------GGTAAC--------

Fig.9. **Schematic representation of various probes used in gel mobility retardation assay and nucleotide sequence of wild and mutant probes.** Number of each probe indicate the sites of 5' and 3' end of the probes from the transcriptional start site. Dotted lines indicate sequences identical to upper sequence.

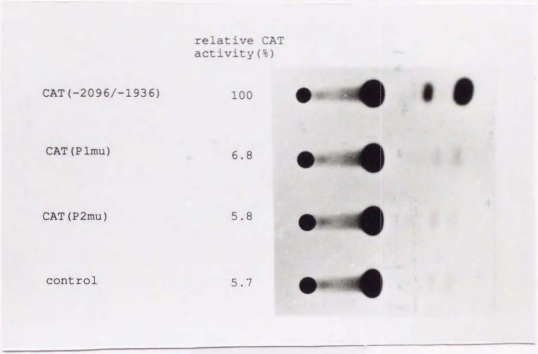


Fig. 10. **CAT activities of mutant reporter plasmids in muscle cells.** Mutationts are introduced in pLCCAT(-2096/-1936). Nucleotide sequence of mutants are shown in Fig. 9. The CAT activity of the each construct relative to pLCCAT(-2096/-1936) is indicated. The plasmids, CAT(P1mu) and CAT(P2mu), are mutant plasmids, where mutations were introduced in P1 or P2 site of ELC1, respectively.

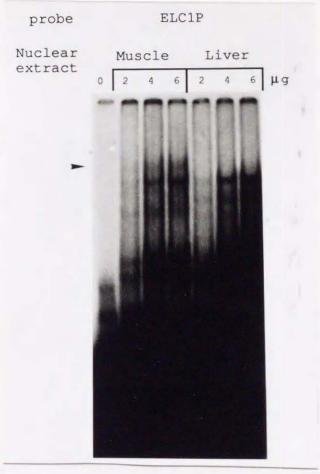


Fig. 11. **Detection of a nuclear protein that binds to the P-region of ELC1 by a gel mobility retardation assay.** Nucleotide sequence of probe ELC1P (-2005 to -1953) is shown in Fig. 9. Nuclear extracts are prepared from the breast muscle or the liver of 12- and 13-day-old chicken embryos. The arrow indicates a muscle-specific retarded band.

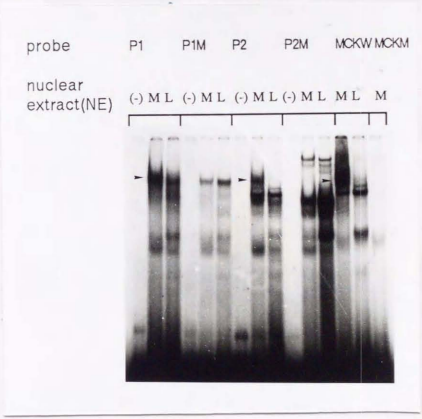


Fig. 12. **Gel mobility retargation assay.** Nucleotide sequence of each probe is shown in Fig. 9. Nuclear extracts are prepared from the breast muscle (M) or the liver (L) of 12- and 13-day-old chicken embryos. The arrows indicate muscle specific retarded bands.

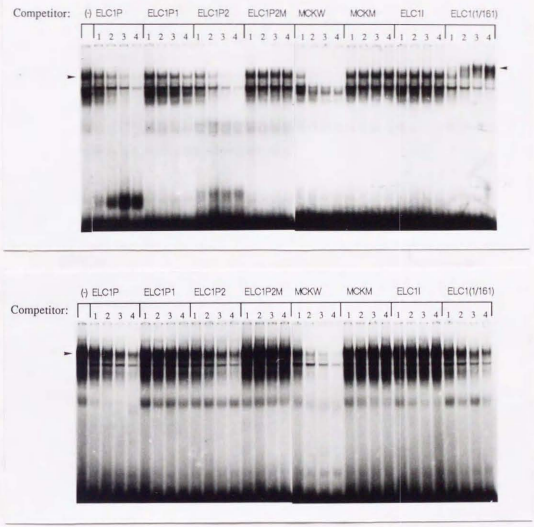


Fig.13. **Sequence specificity of P2/MCK binding.** (a) Probe P2 are used with the muscle nuclear extract. Competitors, which are shown in Fig. 9, were added, at a 40- (lane 1), 200- (lane 2), 400- (lane 3), or 800- (lane 4) fold molar excess of labeled probes. The arrows indicate specific retarded bands. (b) Probe MCK are used. Other conditions are the same as (a).

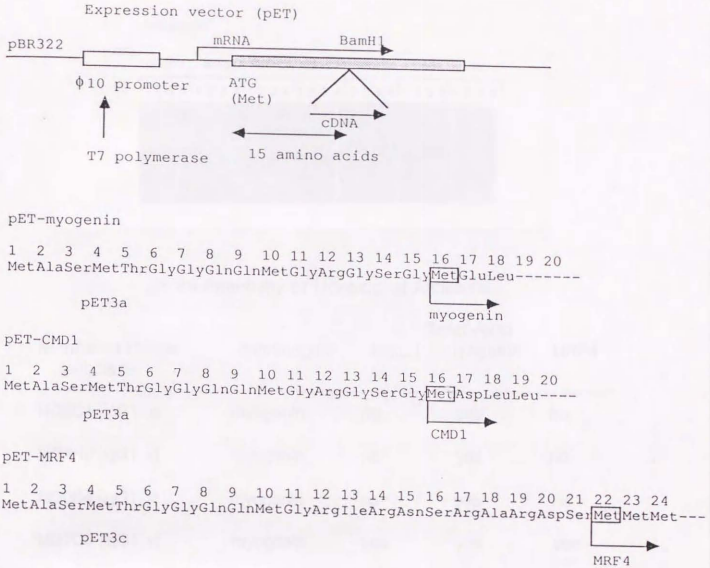


Fig. 14. **Construction of procaryotic expression plasmids, pET-myogenin, pET-CMD1, pET-MRF4 and their expression.** Respective cDNAs linked BamHI or BglII linkers were inserted to BamHI site of pET vectors (53) in correct reading frame. Expressed proteins contain 15 to 21 amino acids exess from the amino terminus methionin derived from the gene 10 of the T7 phage capsid protein gene.

Cross Reactivity of Monoclonal Antibodies

Number of MoAbs (subclass)	immunogen	Reactivity to CMD1	myogenin	MRF4
MG5C1 (IgG1, κ)	myogenin	no	yes	no
MG4H2 (IgG1, κ)	myogenin	no	yes	no
MG6B4 (IgG1, κ)	myogenin	no	yes	no
MG7C6 (IgG1, κ)	myogenin	yes	yes	yes
MG1C12 (IgG1, κ)	myogenin	yes	yes	no
CD6B6 (IgG1, κ)	CMD1	yes	no	no

Fig. 15. **Determination of crossreactivity of monoclonal antibodies by immunoblotting.** Crossreactivity of monoclonal antibodies against expressed proteins in E. coli, including CMD1 (lane 1), myogenin (lane 2), MRF4 (lane 3), and control 1DMC (lane 4) were determined by SDS polyacrylamide gel electrophoresis and immunoblotting. The data are indicated in Table.

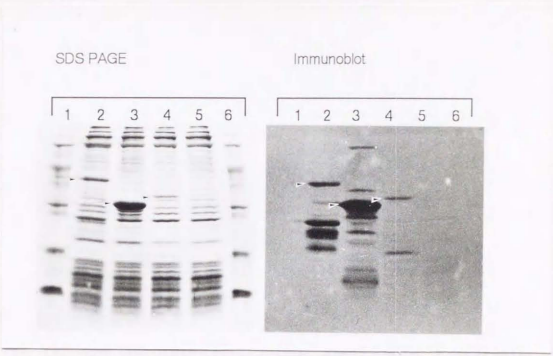


Fig. 16. **SDS PAGE and immunoblot analysis of expressed proteins in E. coli.** Respective lysates, containing CMD1 (lane 2), myogenin (lane 3), MRF4 (lane 4), and control 1DMC (lane5) were immunoblotted. Lane 1, 6 indicates MW marker (Bio-Rad). The arrows indicates respective proteins.

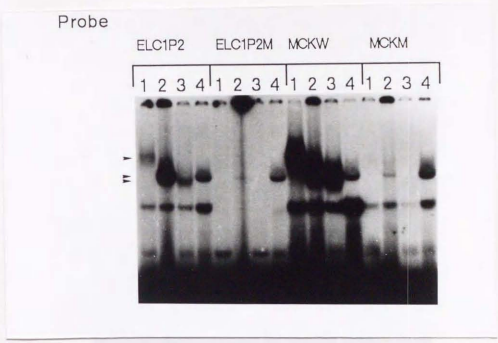


Fig. 17. **Gel mobility retardation assay with precaryotic expressed proteins.** Using respective proteins expressed in E. coli, including CMD1 (lane 1), myogenin (lane 2), MRF4 (lane 3), and control 1DMC (lane 4), a gel mobility retardation assay was performed with respective probes listed in figure. The Arrows indicate specific retarded bands.

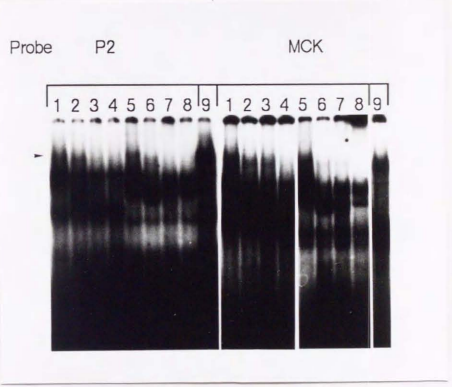


Fig. 18. **Gel mobility retardation assay with proteins expressed in E. coli mixed with liver nuclear extract.** Expressed proteins in E. coli, including CMD1 (lane 1, 5), myogenin (lane 2, 6), MRF4 (lane 3, 7) , or control 1DMC (lane 4, 8), were mixed with liver nuclear extract (lane 1, 2, 3, 4) or primary cultured fibroblasts nuclear extract (lane 5, 6, 7, 8) or muscle nuclear extract alone (9). The gel retardation assay was performed with probes, P2 or MCK enhancer. The arrow indicates a new retarded band.

Fig. 19. **Competitive gel mobility retardation assay.** CMD1 (lane 1 to 5), and myogenin (lane 6 to 10) were mixed with liver nuclear extract, then the gel mobility retardation assay was performed with P2 as a probe. Unlabeled competitors, including P2 (lane 2, 7), P2M (lane 3, 8), MCK (lane 4, 9), and MCKM (lane 5, 10), were added at a 1000-fold molar excess of the labeled probe. The arrow indicates the retarded band.

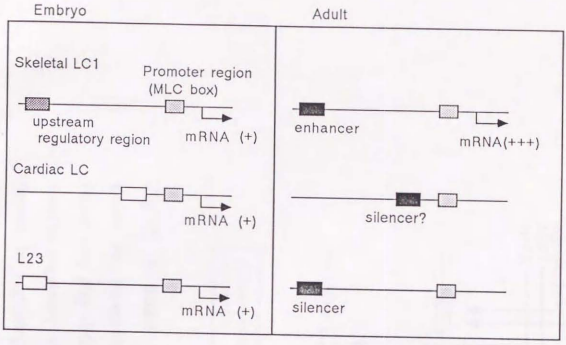


Fig. 20. **A model of coordinate and specific MLC genes expression in embryonic and adult skeletal muscle.** Skeletal MLC1 is expressed in embryonic skeletal muscle owing to the presence of LC1 enhancer. Cardiac and embryonic MLCs are expressed in embryonic skeletal muscles owing to the presence of MLC boxes (57). In adult skeletal muscles, the skeletal MLC is active owing to the presence of the MLC1 enhancer (51, 26), while cardiac (Uetsuki et al., personal communication), and embryonic (57) MLCs are inactivated owing to the presence of negative regulatory elements.

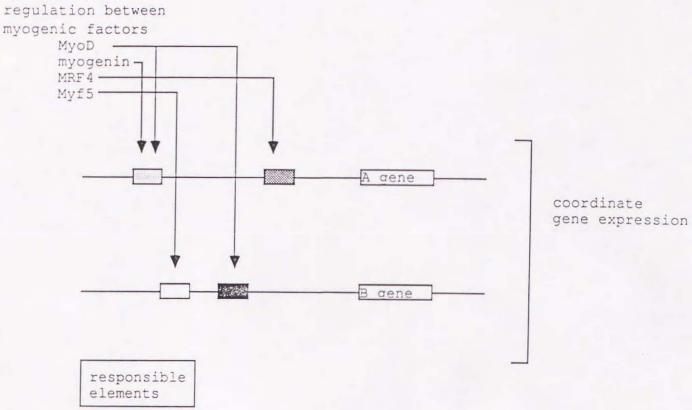
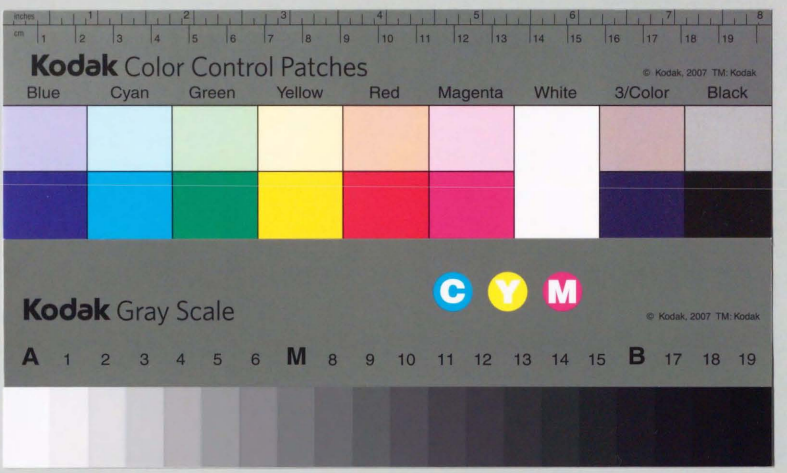


Fig. 21. **A model of coordinate expression of muscle specific genes by myogenic determination factors.** Each myogenic factor may play different roles in activation of several muscle specific regulatory elements by directly binging, but myogenic factors, by regulating with one another to make a cascade or a network (52), may lead to a coordinate expression of muscle specific genes.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