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Upstream Regulatory Region for Inducible Expression of the Chicken Skeletal Myosin Alkali Light-Chain Gene

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The expression of the fast type of myosin alkali light chain 1 is induced during the differentiation of muscle cells. To study the mechanism of its gene regulation, we joined the sequence of the 5′-flanking and upstream region of the chicken myosin alkali light-chain gene to the structural gene for chloramphenicol acetyltransferase (CAT). The fusion gene was introduced either into quail myoblasts transformed by a temperature-sensitive mutant of Rous sarcoma virus (*ts*NY68) or into chicken myoblasts, and the transiently expressed CAT activity was assayed after the differentiation of the myoblasts. From the experiments with the external and internal deletion mutants of the fusion gene, the *cis*-acting regulatory region responsible for the enhanced expression of the CAT activity in response to the cell differentiation was found to be localized at 2 kilobases upstream of the transcription initiation site. This region of 160 nucleotides contained two pairs of short sequences worthy of note, a direct repeat of 12 nucleotides, and an inverted repeat of 8 nucleotides. The nucleotide sequences of the 5′-flanking sequence up to nucleotide −3381 were determined and compared with those of the upstream activating elements of actin genes.

cis-Acting regulatory DNA elements have been identified for the expression of eucaryotic genes (for a review, see reference 29). The TATA box is necessary for accurate initiation of transcription, and the CCAAT box and CCGCCC element control constitutive transcription. In addition to promoter elements, enhancer or regulatory elements are located at various distances upstream or downstream of the promoter. These elements are specifically involved in the inducible expression of genes for heat shock proteins (3), metallothionein (44), β-interferon (16, 18), c-*fos* (49), and P-450c (46) and in the tissue-specific and developmental stage-specific expression of genes such as those for immunoglobulin (21) and β-globin (7). Specific *trans*-acting factors are known to bind these *cis*-acting elements to regulate the transcription (13).

In the process of skeletal muscle development, mononucleated myoblasts cease to proliferate and fuse with one another to form multinucleated myotubes. During this terminal differentiation, expression of the genes specific for muscle cells is induced; these genes encode many kinds of contractile proteins including the fast type of myosin alkali light chain 1, α-actin, myosin heavy chains, tropomyosin, and troponin (5, 8, 10, 11, 17, 45, 51). This iduction was also observed with other muscular enzymes such as creatine kinase (14, 27). From observations that the induction of these contractile proteins is brought about simultaneously in primary cultured myoblasts, it was suggested that the transcriptional regulation of their genes are coordinated with one another in association with differentiation (10, 11). However, the molecular mechanism underlying the coordinate regulation remains to be elucidated. As a first step to understanding the regulatory mechanism, it is important to identify the *cis*-acting DNA elements responsible for inducible expression of these genes. DNA transfection experiments with the fusion genes, which consist of various test DNA fragments and a reporter gene, are useful for this purpose. So far, the inducible expression of fusion genes has been found in transient expression experiments with genes for α-actin (20), cardiac actin (32), heart myosin light chain 2A (52), and creatine kinase (23), and also in stable transformation systems (24, 30, 37). However, a detailed analysis of the upstream regulatory regions was done only with actin genes (2, 32, 33).

Recently, several research groups including ours isolated genes for the skeletal muscle type of myosin alkali light chains ($MLC1_f$/$MLC3_f$) from chickens (36), rats (38), and mice (40) and determined their nucleotide sequences. The results showed that two promoters for $MLC1_f$ and $MLC3_f$ were present in a single gene and were separated by a long intron (9 to 11 kilobases). The two promoters of this gene are separately regulated (47), so that the expression of $MLC1_f$ and $MLC3_f$ does not take place at the same time during muscle development (1).

To elucidate the regulatory mechanisms of the $MLC1_f$/$MLC3_f$ gene during the differentiation of muscle cells, we constructed a fusion gene by ligating the possible regulatory region of the 5′-upstream flanking sequence of $MLC1_f$/$MLC3_f$ gene to a reporter gene, the chloramphenicol acetyltransferase (CAT) gene, and transfected it into cultured cells to identify the *cis*-acting regulatory elements. In this paper we report that the expression of the fusion gene was activated in association with the differentiation of the Rous sarcoma virus (RSV) *ts*NY68-transformed quail muscle cells (34) and chicken primary myoblast cells. A regulatory DNA element necessary for the gene activation is located at about 2 kilobases upstream from the transcription initiation site, and the nucleotide sequences in the regulatory region of the $MLC1_f$ and actin genes are compared.

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MATERIALS AND METHODS

Construction. $MLC1_f/MLC3_f$-CAT fusion genes (pLCx) were constructed by inserting the 5'-upstream flanking and untranslated region of the chicken $MLC1_f/MLC3_f$ genomic DNA, λSMA3 (36), into the *Sal*I-*Hin*dIII site of pMLCAT (15) in the correct orientation. In pLC3381, the insert spanned the region from the *Bgl*II site (base −3381 with respect to the transcription start site) to the *Hin*fI site (base +63) in the leader sequence, and its 5' and 3' ends were converted into *Sal*I and *Hin*dIII sites, respectively, by linker ligation. For the fusion genes with various external deletions, the 5' upstream fragments of the $MLC1_f/MLC3_f$ gene were digested with *Bal*31 exonuclease for various periods or with appropriate restriction endonucleases. The shortened fragments of the $MLC1_f/MLC3_f$ gene were inserted again into the same site of pMLCAT after ligation of the *Sal*I linker to the 5' end. A *Hin*dIII fragment from −2241 to −1744 was deleted from pLC3381 to produce an internal deletion mutant, pLCΔH.

DNA transfection and cell cultures. Transfection was carried out by the calcium phosphate method (50) with 5 μg of plasmid DNA per dish 1 day after plating, and then the cells were treated with 10% (vol/vol) glycerol for 2 min.

Quail myoblasts transformed by RSV *ts*NY68 were originally prepared and kindly provided by D. Montarras and M. Y. Fiszman (34). The cells were maintained and propagated in Dulbecco modified Eagle medium with 10% fetal calf serum at a temperature permissive for viral transformation (35°C). For the induction of cell differentiation, the temperature was shifted to a nonpermissive temperature (41°C). The day before transfection, the transformed quail myoblasts were plated at a density of 10^6 cells per 60-mm gelatin-coated dish. At 1 h after glycerol treatment, one set of dishes were incubated at 41°C and the rest remained at 35°C as controls. The cells were harvested for assay of the expressed CAT activity 3 days after the shift of the temperature.

Primary cultures of chicken myoblasts were prepared from breast muscles of 12-day-old embryos by brief enzymatic digestion with trypsin (0.05% [wt/vol]) at room temperature for 10 min and subsequent pipetting. Isolated cells (10^6 per 60-mm gelatin-coated dish) were grown in Eagle minimal essential medium containing 10% horse serum and 2% chicken embryonic extracts. The cells were cultured for 12, 24, 48, and 84 h after transfection.

Chicken embryo fibroblasts, which were isolated from the primary cultured myoblasts, and Hepa-1 cells, a cell line derived from mouse hepatoma, were propagated in Eagle minimal essential medium containing 10% fetal calf serum. These cells were harvested 2 days after transfection.

CAT assay. The CAT activity was assayed as described previously (19). All assays were performed within the range of a linear relation of the activities with the time of incubation or the concentration of extracted proteins.

Extraction and analysis of cellular RNA. For the investigation of transcripts from fusion genes, total RNAs of cultured cells were extracted with 4 M guanidine thiocyanate (6).

RNase mapping of fusion gene transcripts was performed as described in a previous report (31). The isolated RNA (20 μg) was hybridized with an uniformly labeled RNA probe (3.3×10^5 cpm) at 45°C for 11 h and digested with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) at 30°C for 1 h. For the hybridyzation probe, the *Ava*I-*Eco*RI fragment of pLC3381 was subcloned into the polylinker site of pSP65 (31). After being linearized by *Pst*I, the plasmid DNA was transcribed by SP6 polymerase to be uniformely labeled with [α-^{32}P]UTP. The probe was 459 nucleotides long and included the sequence from the *Eco*RI site in the CAT structural gene to the nucleotide 114 upstream of the cap site in the $MLC1_f/MLC3_f$ gene. The protected probes of RNase mapping analysis were analyzed by electrophoresis in a 6% polyacrylamide gel containing 7 M urea. The gels were dried and autoradiographed at −80°C for 3 days.

Northern blot analysis. Northern (RNA) blot hybridization was performed as described by Thomas (48). Total RNA (5 μg) from each sample was fractionated by electrophoresis in an agarose gel (0.8%) and blotted onto a nitrocellulose filter. For the detection of mRNA coding for quail MLC1s, a filter was hybridized with nick-translated chicken $MLC1_f$ cDNA (pSMA1-1) (35) at 41°C overnight. The hybridization filter was washed twice at 50°C for 30 min with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% sodium *N*-lauroyl sarcosinate and autoradiographed for 5 h at −80°C.

DNA sequence analysis. DNA sequences were determined by the chain termination method as described previously (43). Database searching was carried out by using a sequence analysis program, GENAS (25), with the DNA databases EMBL (release 7.0) and GenBank (release 40.0) in a FACOM M382 computer at Kyushu University.

RESULTS

Expression of the endogenous $MLC1_f/MLC3_f$ gene in transformed quail myogenic cells. Quail myoblast cells transformed with RSV *ts*NY68 were used for transient-expression experiments. Differentiation of the cells is known to be controlled by a temperature-sensitive mutant of RSV (*ts*NY68) (34). When shifted to a nonpermissive temperature (41°C), the cells start to fuse simultaneously within 24 h after the shift of temperature and form large myotubes in 2 or 3 days. During the differentiation of the cells, the synthesis of muscle-specific proteins including the adult fast type of $MLC1_f$ is increased to reach a maximum after 5 days of the experiment (34). Since the kinetics of its mRNA accumulation had not been known, the change in the content of MLC1 mRNA in this process was investigated by Northern blot analysis with the cloned chicken $MLC1_f$ cDNA (pSMA1-1) as a probe (35). The synthesis of the $MLC1_f$ mRNA was induced by a shift to the nonpermissive temperature, and the mRNA level reached a plateau level within 24 h (Fig. 1). Thereafter, the level of $MLC1_f$ mRNA was steady at least for 5 days (data not shown). This result indicated that the induction of endogenous $MLC1_f/MLC3_f$ gene expression was strictly controlled in association with the differentiation induced by the temperature sensitivity of the viral gene product.

Transfection of the fusion gene into RSV *ts*NY68-transformed quail myoblasts. The construction of the chicken $MLC1_f/MLC3_f$-CAT fusion genes is summarized in Fig. 2. A fusion gene, pLC3381, contained part of the 5'-flanking sequence (3,381 bases) and consecutive 5'-nontranslated sequence (63 bases) of the $MLC1_f/MLC3_f$ gene. Therefore, the promoter and leader sequence for the $MLC3_f$ mRNA were not contained in this construction (36). Plasmid pLC3381 was transfected into the quail myoblasts by the calcium phosphate method. Half of the cells were maintained at a permissive temperature, and the other half were cultured at a nonpermissive temperature to allow differentiation of the cells. As a positive or negative control, the cells were transfected with either pSV2CAT (19) or pMLCAT (15)

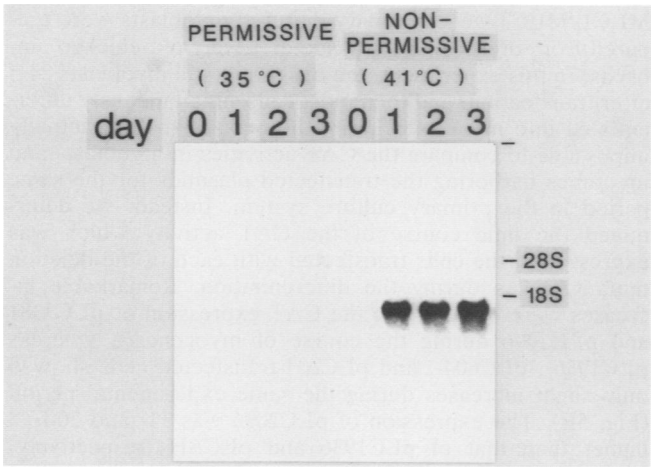

FIG. 1. Expression of the endogenous $MLC1_f/MLC3_f$ gene during the differentiation of RVS *ts*NY68-transformed quail myoblasts. The myoblasts (34) were allowed to proliferate at a permissive temperature (35°C) for viral transformation or to differentiate to myotubes at a nonpermissive temperature (41°C). Total cellular RNAs were prepared from differentiating myotubes before (day 0) and 1, 2, and 3 days after the shift of the temperature and from proliferating myoblasts for a control. A 5-μg sample of total RNA was loaded for electrophoresis and hybridized with the nick-translated cDNA of chicken $MLC1_f$ (35).

DNA, and thereafter they were treated similarly. pMLCAT contained pML sequence (28) ligated directly to the 5′ end of the CAT structural gene (Fig. 2), thus lacking any promoter elements for the CAT gene. To compare the CAT activities in myoblasts and myotubes and to minimize variations between experiments, the CAT activities were normalized against that of pSV2CAT in each experiment and represented as percentages of that in the pSV2CAT-transfected cells. The CAT activity, which was slightly expressed in the quail myoblasts, was enhanced during cell differentiation (Fig. 3). The enhanced CAT activity in myotubes was 6.4% of the standard and 4.4-fold higher than that in myoblasts.

RNase mapping of the fusion gene transcripts. RNase mapping methods were used to determine the transcription start site of the fusion gene. The uniformly labeled single-stranded RNA, which contained the sequence from the *Eco*RI site in CAT structural gene to the *Ava*I site in the 5′-flanking sequence of $MLC1_f/MLC3_f$ gene (Fig. 4), was hybridized with total RNA prepared from the quail myotubes or myoblasts transfected with pLC3381 and then digested with a mixture of RNase A and RNase T_1. Subsequent analysis of the digested RNA by polyacrylamide gel electrophoresis revealed one major protected band of 326 nucleotides, together with a minor one of 329 nucleotides in the RNA from the myotubes (Fig. 4). The length of the major band coincided with the length which was expected when the correct transcription start site was used. We detected only a slightly protected band at the equivalent position in the myoblast RNA. Densitometric analysis of the autoradiograph showed that the protected band of 326 nucleotides increased more than 20-fold during the differentiation of myoblasts. We conclude, therefore, that the enhanced CAT activity observed in the myotubes reflects the induced transcription of the transfected fusion gene in association with the cell differentiation, thus mimicking the expression of the endogenous $MLC1_f/MLC3_f$ gene.

Transfection of various deletion mutants derived from the fusion gene pLC3381. To localize the functional regions responsible for the induction in the 5′-flanking sequence of the $MLC1_f/MLC3_f$ gene, we constructed a set of hybrid plasmids with various external deletions (Fig. 2). These fusion genes with external deletions were transfected into the cells to assay the inducible expression. The results of the

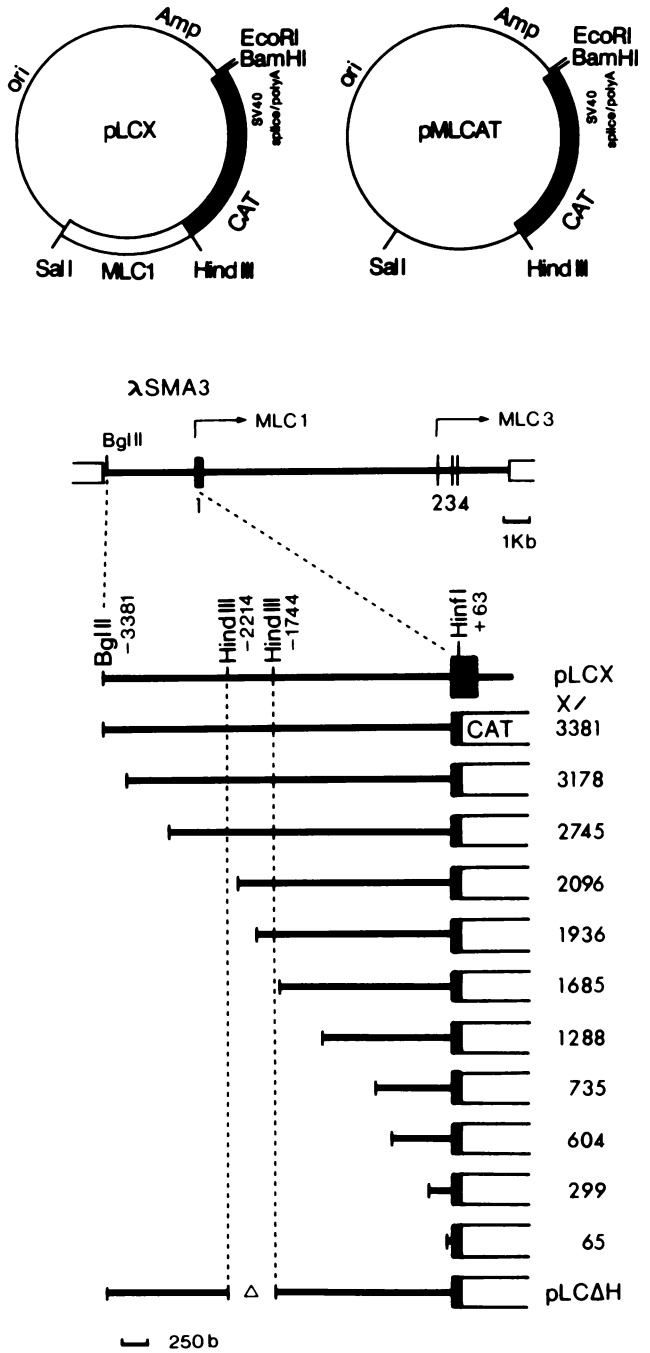

FIG. 2. Structure of the $MLC1_f/MLC3_f$-CAT fusion genes. The DNA fragment of the upstream and consecutive untranslated region was excised from genomic clone DNA of the chicken $MLC1_f/MLC3_f$ gene, λSMA3 (36), and inserted into the *Sal*I-*Hin*dIII site of pML-CAT (15) to construct the fusion genes (pLCX and pLCΔH). X indicates the length of the 5′-flanking sequence of the $MLC1_f/MLC3_f$ gene from the transcription start site. The inserted fragments had a common 3′ end (+63). Δ, Region of an internal deletion. SV40, Simian virus 40.

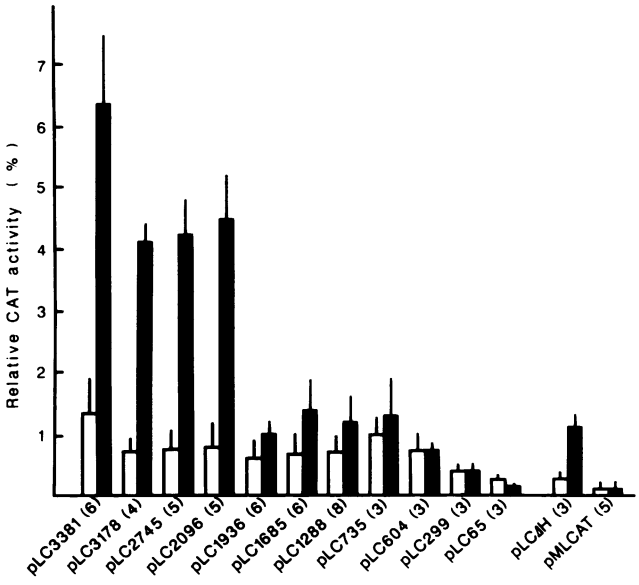

FIG. 3. Regulatory regions in the 5'-flanking sequence of the $MLC1_f/MLC3_f$ fusion gene for inducible expression during the differentiation of myoblasts. The figure shows a diagram of the relative CAT activities expressed in RSV *ts*NY68-transformed quail myogenic cells transfected with the $MLC1_f/MLC3_f$-CAT fusion genes. The cell extracts were prepared 3 days after transfection, and 10 μg of protein was used for the assay. The CAT activity was expressed as a percentage of the activity obtained with pSV2CAT (19). Results are the averages of the CAT activities from more than three experiments (numbers of experiments shown in parentheses). Bars indicate mean values ± standard deviation. Symbols: ▭, myoblasts cultured at 35°C; ▬, myotubes differentiated at 41°C.

experiments clearly showed that external deletions of two regions, from −3381 to −3178 and from −2096 to −1936, reduced markedly the CAT activity expressed in the myotubes (Fig. 3). In particular, the deletion from −2096 to −1936 caused a 4.5-fold decrease in the expressed activity and resulted in a lower ratio of the activities in myotubes and myoblasts (from 6.4- to 1.6-fold). Further deletions up to position −735 had little effect on the expressed CAT activity, and the consecutive deletions beyond this position resulted in a gradual loss of the CAT activity in the myotubes as well as in the myoblasts. This indicated that the sequence from −735 to the transcription start site may be involved in the basal level of expression, as is the case with most of the eucaryotic genes (4, 12, 22).

To ascertain that a region between −2096 and −1936 was necessary for the activated expression in response to cell differentiation of myoblasts, we constructed an internal deletion mutant (pLCΔH) of plasmid pLC3381 in which a region from −2214 to −1744 was deleted. As expected, the CAT activity in the myotubes transfected with pLCΔH was lowered to the level of the activity in pLC1936-transfected cells. This result confirmed that the sequence responsible for the induction of $MLC1_f/MLC3_f$ gene expression was localized in a region of 160 nucleotides between −2096 and −1936.

Expression of the CAT activity in primary cultured cells of chicken muscle. To ascertain whether the activation of the fusion gene, which was observed in the RSV-transformed myogenic cells, actually occurs in normal cultured cells, we investigated the expression of fusion genes in primary cultured cells of chicken muscle, the homologous cells for the $MLC1_f/MLC3_f$ gene. Primary cultured myoblasts were prepared from breast muscle tissue of 12-day-old chicken embryos. In this experiment, the cells were still myoblasts 24 h after transfection, and then most cells simultaneously differentiated into myotubes during the next 24 h. It is actually impossible to compare the CAT activities in myoblasts and myotubes harboring the transfected plasmids for the same period in the primary culture system. Instead, we determined the time course of the CAT activity which was expressed in the cells transfected with each of the deletion mutant DNAs during the differentiation. Remarkable increases were observed in the CAT expression of pLC3381 and pLC2096 during the course of myogenesis, whereas pLC1936-, pLC604-, and pLCΔH-transfected cells showed only slight increases during the same experimental period (Fig. 5B). The expression of pLC2096 was 11- and 20-fold higher than that of pLC1936 and pLCΔH, respectively. These observations lead us to the same conclusion as that drawn from the experiment with RSV-transformed quail cells, i.e., that the region from −2096 to −1936 is responsible for the activation of the $MLC1_f/MLC3_f$ gene in association with the differentiation of muscle cells.

Primer extension analysis of the RNA preparations from

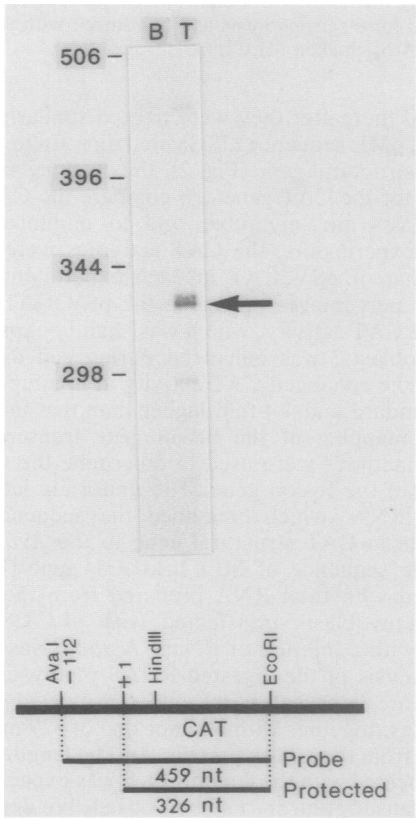

FIG. 4. RNase mapping of the transcription start sites in the fusion gene. Fusion gene pLC3381 was transfected into RSV *ts*NY68-transformed quail myoblasts, and the cells were allowed to proliferate at 35°C or to differentiate at 41°C for 3 days. Total cellular RNAs were prepared and subjected to RNase mapping analysis. A uniformely labeled probe was 459 nucleotides (nt) long and contained a region which extended from the *Eco*RI site in the CAT structural gene to the *Ava*I site (−112) in the 5'-flanking region of the $MLC1_f/MLC3_f$ gene. The protected fragment was expected to be 326 nucleotides long if the correct start site for the $MLC1_f$ mRNA was used. Lanes: B, RNA prepared from myoblasts; T, RNA prepared from myotubes.

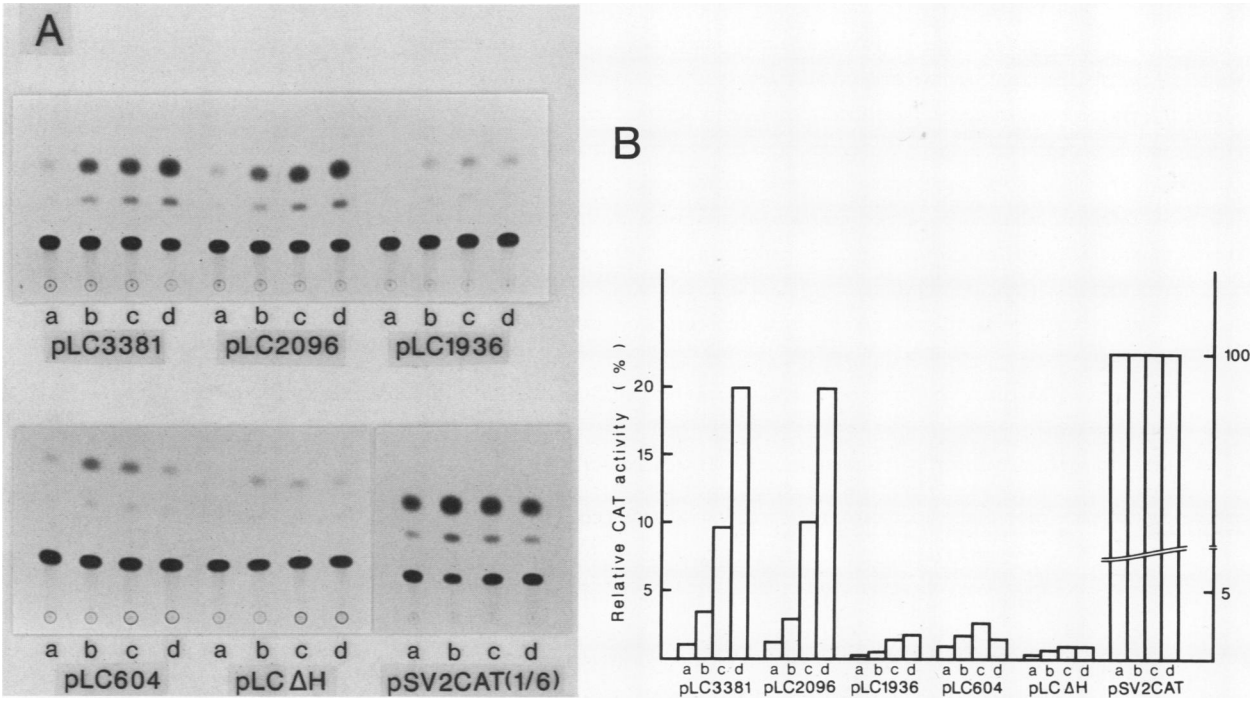

FIG. 5. Expression of the fusion genes in chicken primary muscle cell cultures. (A) The CAT activities in the differentiating myoblasts transfected with pLC3381, pLC2096, pLC1936, pLC604, pLCΔH, and pSV2CAT. The cells were harvested at 12 h (a), 24 h (b), 48 h (c), or 84 (d) after transfection. For the CAT assay, 30 μg of the protein extracts was used and the reactions were carried out at 37°C for 1 h. For the assay of pSV2CAT-transfected cells, the reactions were carried out for 20 min with 15 μg of protein. The cells were still myoblasts at 12 and 24 h after transfection and then formed myotubes at 48 and 84 h. (B) Diagram of the CAT activities normalized against pSV2CAT.

the cells transfected with various fusion genes as described in Fig. 5 indicated that the correct transcriptional initiation site was also used in the primary cell culture and that the amounts of the transcripts estimated here were in proportion to the CAT activities (data not shown).

Expression of the CAT activity in nonmyogenic cells. A cell type specificity in the expression of the fusion gene was investigated by transfecting pLC3381 and pLC1936 DNAs into various nonmyogenic cells including chicken embryo fibroblasts and Hepa-1, COS, and HeLa cells. The two fusion genes were expressed similarly at low levels in chicken embryo fibroblasts and Hepa-1 cells (Fig. 6). The CAT activities in chicken embryo fibroblasts transfected with pLC3381 and pLC1936 DNAs were 1.5 and 0.76% of that of pSV2CAT, and the activities in Hepa-1 cells were 1.4 and 1.0% of that of pSV2CAT, respectively. Even lower activities were observed in HeLa and COS cells transfected with the two plasmid DNAs (data not shown). These results indicated that the *cis*-acting DNA element in the 5′ upstream region of the $MLC1_f$ gene exerts its enhancing effect in a cell type-specific manner.

Sequence analysis of 5′ upstream region. We determined the nucleotide sequence of the 5′ upstream region flanking the $MLC1_f/MLC3_f$ gene (Fig. 7). A close survey of the sequence allowed us to find interesting DNA elements in the region (−2096 to −1936); deletion of these elements caused a marked reduction in the gene expression in response to the differentiation. In this region we found an incomplete direct repeat of 12 nucleotides (10 matches out of 12) and an inversely oriented repeat of 8 nucleotides just downstream of the direct repeat (Fig. 7). No identical sequences to these elements, but one similar to the 12-base sequence at −957, were found elsewhere in the 5′-flanking sequence.

DISCUSSION

We demonstrated that a $MLC1_f/MLC3_f$-CAT fusion gene (pLC3381) was expressed in the RSV *ts*NY68-transformed quail myogenic cells and that its expression was enhanced in association with the differentiation of the cells. This fusion gene was also expressed more efficiently (about fivefold) in chicken primary culture of muscle cells than in the quail myotubes, whereas in nonmyogenic cells its expression was as low as that in the quail myoblasts or even lower. From these results we considered that the enhancement of the fusion gene expression in the differentiating quail cells

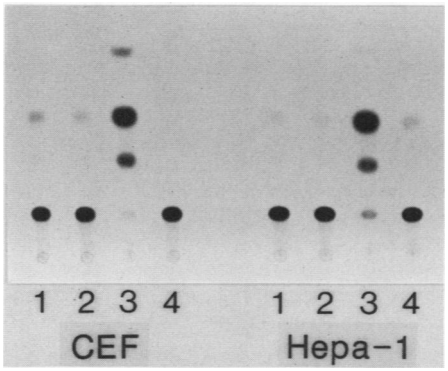

FIG. 6. Expression of the fusion genes in nonmyogenic cells. The fusion genes pLC3381 and pLC1936 were transfected into chicken embryo fibroblasts (CEF) and Hepa-1 cells. The cells were harvested 2 days after transfection. The CAT activities were quantified by using 10 μg of protein extracts and a 1-h incubation at 37°C. Lanes: 1, pLC3381; 2, pLC1936; 3, pSV2CAT; 4, pMLCAT.

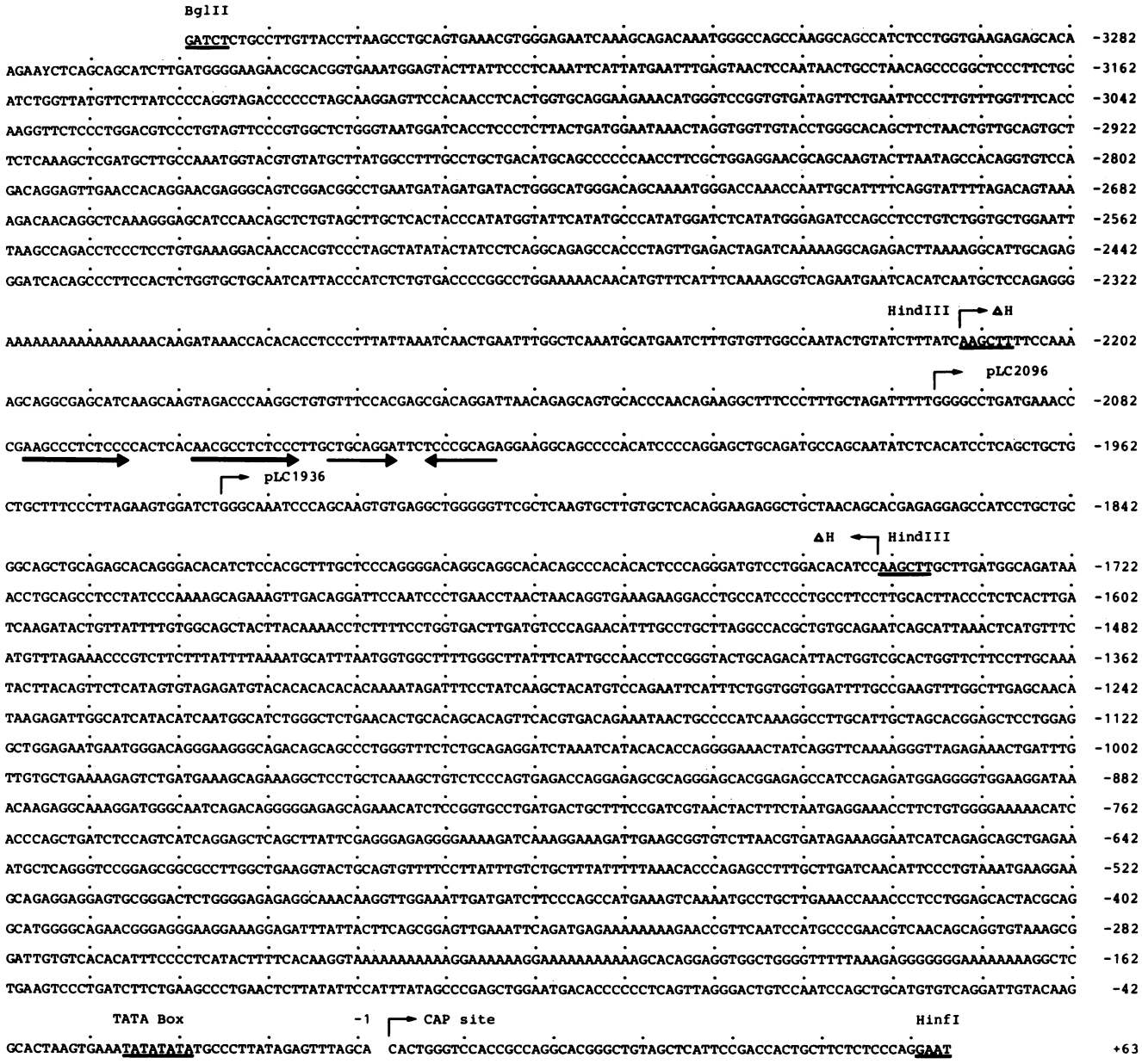

FIG. 7. Nucleotide sequence of the 5′-flanking region of the chicken $MLC1_f/MLC3_f$ gene. The sequences were determined by the chain termination method with the M13 vector. Almost all the sequences were determined in both directions. Nucleotides are numbered as negative from the transcription start site. Large arrows show the position of direct and indirect repeats found in the regulatory region responsible for the enhanced expression in the cell differentiation. The 5′ ends of fusion genes pLC2096 and pLC1936 are indicated by arrows. The region of the internal deletion in pLCΔH is shown by the arrows with ΔH.

mimicked faithfully the induction of the endogenous $MLC1_f/MLC3_f$ gene expression in the process of myogenesis.

The results of experiments with a series of deletion mutants in the 5′ upstream region of the fusion genes tracked down the three main regions necessary for the fully activated expression of the gene in myogenesis, that is, the sequences from −3381 to −3178, from −2096 to −1936, and from −735 to −65 (Fig. 3). Of these elements, the region from −2096 to −1936 appears to be most important in regard to the inducibility of the gene in response to cell differentiation, because either internal or external deletion of this region resulted in the loss of a large part of the activated expression in both quail and chicken myotubes. In this context, it is noteworthy that the two kinds of interesting short sequences are present in this region: one (12 nucleotides) is in a direct repeat, and the other (8 nucleotides) is in an inverse repeat, although the role of these sequences in the regulatory mechanism of gene expression remains to be studied. The sequence from −3381 to −3187 appeared to be necessary for the fully induced activation of the gene, but may be subsidiary, because its enhancing effect could not be practically exerted without the sequence from −2214 to −1744 (pLCΔH) in both quail and chicken myotubes. The sequence proximal to the transcription start site (−735 to −65) was necessary for the basal level of expression. In conclusion, the upstream regulatory ele-

ment(s) for the differentiation-dependent expression of the $MLC1_f/MLC3_f$ gene was located in the region from −2096 to −1936.

To further characterize the region from −2096 to −1936, we constructed the fusion genes with a heterologous promoter by inserting the upstream regions into pA10CAT (26) and investigated the expression in primary cell cultures of chicken myotubes. However, the insertion of the regulatory region (−2096 to −1936) or most of the upstream region (−3381 to −112) did not activate the transcription driven by the early promoter of simian virus 40. The upstream regulatory element(s) of $MLC1_f/MLC3_f$ gene may be a promoter-specific enhancing element.

Most of the contractile protein genes are activated coordinately in the process of myogenesis (10, 11). This suggests that the regulatory regions of these genes share a common *cis*-acting element to induce the enhancement of transcription. Recent studies with transient-expression experiments revealed the regulatory regions of two actin genes. The regulatory region of the chicken α-actin gene is located in the region from the transcription initiation site to −200 (2), and the region from −101 to −485 in the human cardiac actin gene is sufficient for the high level of the regulated expression (32). We compared the nucleotide sequences in these regulatory regions of actins and that of the chicken $MLC1_f/MLC3_f$ gene (−2096 to −1936). No homologous sequence was shared by the regulatory regions of the three genes, which were equally responsible for the inducible expression of the genes during the differentiation of the myoblast. No sequences similar to the repeated sequences in the regulatory region of $MLC1_f/MLC3_f$ gene were found in the actin genes, and the CArG motif, which is the regulatory element of the human cardiac actin gene (32, 33), was not found in the $MLC1_f/MLC3_f$ gene. We cannot exclude the possibility that a common *cis*-acting regulatory element is located outside the analyzed region or that a *trans*-acting factor recognizes both of the regulatory elements with different sequences to activate the expression of these genes simultaneously, as is reported with the regulation of yeast *CYC1* and *CYC7* genes (39). It may not be surprising, however, that a common *cis*-acting DNA element was not found in the regulatory region of the three genes to substantiate their apparent coexpression in connection with myogenesis. It is known that the $MLC1_f/MLC3_f$ and α-actin genes are not expressed coordinately in all skeletal muscle cells. $MLC1_f$ is not synthesized in some slow-twitch muscles (type I fibers) (42) and the rat muscle cell line L6 (41), whereas the α-actin gene is activated in these cells. This may suggest that a regulatory mechanism of the $MLC1_f/MLC3_f$ gene is different from that of the α-actin gene. More complex regulatory mechanisms may work to cause the coexpression of the muscle proteins during myogenesis.

In any case, in addition to the $MLC1_f/MLC3_f$ and actin genes, detailed analysis of the regulatory elements and the identification and characterization of the *trans*-acting factors for the other muscle protein genes is necessary for a better understanding of the molecular basis of coordinate regulation during the terminal differentiation of muscle cells.

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