Volume 16 Number 6 1988 Nucleic Acids Research

The promoter of the chicken cardiac myosin light chain 2 gene shows cell-specific expression in transfected primary cultures of chicken muscle

H.H.Arnold*, E.Tannich and B.M.Paterson

Department of Toxicology, Medical School, University of Hamburg, Grindelallee 117, D-2000 Hamburg 13, FRG and [1]Department of Biochemistry, National Cancer Institute, Bethesda, MD, USA

Received January 26, 1988; Accepted February 25, 1988

ABSTRACT

Transcriptional regulation of the chicken cardiac myosin light chain 2 (MLC2-A) gene was investigated in chicken primary myoblast and fibroblast cultures transfected with vector constructs containing the bacterial marker gene for chloramphenicol acetyltransferase (CAT) under the control of the MLC2-A promoter. We here demonstrate that sequences close to the TATA box are sufficient to direct muscle specific and regulated expression of the MLC2-A mRNA. Transcription from MLC2-A promoter/CAT hybrids in myocytes starts from the authentic cap site that is also used in vivo. In primary breast muscle cells, bromodeoxyuridine (BUdR), a reversible blocking agent of cell differentiation, suppresses transcription from the MLC2-A promoter whereas nonmuscle promoters like the RSV- or the cytoplasmic ß-actin promoter are unaffected in their transcriptional capacity. Although the endogenous cardiac MLC2-A gene in chicken is exclusively active in heart, the transfected MLC 2-A promoter escapes this cell type control in primary cultures of breast muscle. These results demonstrate that although muscle specificity of the MLC2-A gene and its transcriptional up-regulation during diffentiation is maintained in a rather short promoter segment, restrictive elements determining the muscle cell type specificity in vivo are either not present in our constructs or are not acting under the conditions of transient transfection.

INTRODUCTION

Differentiation of muscle cells is characterized by the fusion of mononucleated myoblasts to multinucleated myotubes and the concommitent accumulation of muscle specific proteins, such as membrane components, certain enzymes and the contractile proteins which in striated muscle are organized in sarcomers. When cardiac myoblasts differentiate to functional heart cells they do not fuse but accumulate sarcomeric proteins after becoming postmitotic. Different muscle types like slow and fast skeletal muscle or cardiac and smooth muscle synthesize distinct isoforms of contractile proteins transcribed from different genes. The molecular events leading to the formation of muscle structures can be studied in cell cultures which under certain conditions undergo fusion and produce muscle specific proteins. The accumulation of these proteins occurs approximately coordinated with the synthesis of their corresponding mRNAs (1,2). This observation in addition to results from nuclear run-off and DNAse I sensitivity experiments of transcribed muscle genes (3, for review: 4) suggest

that at least part of the rapid increase of muscle specific proteins is due to transcriptional activation of their corresponding genes.

We have previously reported on the isolation and structural characterization of the cardiac myosin light chain 2 (MLC2-A) gene from chicken (5). In the living organism this gene is expressed only in differentiated heart muscle and no indication for a developmental switch exists (6). The structure and location of the putative promoter has been determined earlier (3.7), however, no evidence for its muscle specific function has been presented. In an attempt to define gene sequences involved in tissue specific and differentiation dependent transcription we have addressed the following questions: 1) What are the sequence elements that are implicated in muscle specific transcription in vivo? 2) Are there multiple elements required? 3) Can we minick the transcriptional upregulation of MLC2-A mRNA in differentiating culture systems?

The bacterial chloramphenicol acetyltransferase gene (CAT) has been used to demonstrate tissue-specific promoter activity for a wide variety of genes in several different tissues like insulin and chymotrypsin in pancreas and actins in striated muscle (8-12). In the present paper we describe experiments utilizing the CAT system to characterize sequences upstream of the transcription start site of the MLC 2-A gene which are required for its expression in muscle cells. As recipient cells for transient expression studies of this chicken gene we used established cell lines of mouse and rat origin as well as primary cultures from embryonic chicken breast and heart muscle. Our experiments demonstrate the function of a relatively short upstream MLC2-A sequence that is capable of tissue-specific transcription of the marker gene in muscle cells. This expression is modulated by the drug BUdR which blocks muscle cell differentiation when incorporated into nuclear DNA. The MLC 2-A promoter elements are indiscriminately active in skeletal and cardiac cell cultures when transfected into these cells in contrast to the endogenous gene that is only transcribed in heart cells.

MATERIALS AND METHODS

Cell cultures

Chicken primary muscle cultures have been prepared from 12 day old chicken embryos by dissecting breast muscle free of skin and connective tissue and culturing them under conditions as described in detail elsewhere (13, 14). Primary heart cells were grown from 10 day old embryos according to published procedures (15). For all chicken primary cell cultures BUdR was included in the control medium at a concentration of 5 ug/ml during the entire experiment. The C2C12 mouse muscle cell line was cultured and transfected as previously described (16).

Construction of plasmids containing the chimeric CAT genes

The recombinant plasmids pEMBL-CAT or pUC-CAT containing the bacterial CAT

structural gene and either pEMBL8 vector developed by Dente et al., (17) or pUC8 plasmid were used as recipient vectors for insertion of the promoter containing DNA fragments of the chicken MLC2-A gene. Both vectors are high copy number plasmids derived from pUC8 and contain multiple restriction sites for convenient insertion of test promoters. Plasmid pUC-CAT was constructed in our laboratory by fusion of Hind III pUC8 DNA to the Hind III-Bam HI CAT fragment of 3N-polylinker CAT (generously supplied by P. Gruss, Heidelberg). For the construction of LC-pUC-CAT a 160-bp PstI fragment of the MLC 2-A subclone MLC 14-I (5) was inserted into the Pst site of pUC-CAT and the correct orientation was determined by nucleotide sequence analysis of both cloning junctions using the appropriate primers (18). To generate plasmid LC-RI-CAT the vector pEMBL-CAT was linearized with SmaI and ligated to the 2.5 kb Eco RI fragment of the MLC 2-A gene, that had been treated with DNA polymerase I (Klenow fragment) to obtain flush ends. This gene fragment contains the promoter in the 5'flanking DNA and extends to the Eco RI site in Exon 2 (5). To elongate the 5'flanking region by another 600 nucleotides plasmid 3.1-LC-CAT was constructed by ligation of a 600 nucleotide BAM HI-Sma I fragment into the SmaI site of LC-RI-CAT and recircularizing the chimeric plasmid by blunt end ligation after filling in the BAM HI site.

The LCA-CAT deletions were generated by limited digestion with exonuclease III essentially as described by Henikoff (19) with minor modifications. The 160 base-pairs PstI fragment of LC-pUC-CAT was subcloned into the vector pTZ-18 (Pharmacia) to allow the generation of closely spaced 3'- and 5' protruding ends by the digestion with restriction endonuclease KpNI and BamHI, respectively. In a typical experiment 20 μg of DNA were incubated in a total volume of 100 μl with 450 units of exonuclease III (Boehringer, Mannheim F.R.G.) for time intervalls of 0,2 to 2 min at 25°C. The remaining single stranded DNA was removed by exonuclease VII treatment followed by a short incubation with Klenow fragment of DNA polymerase and the addition of BamHI linkers (Pharmacia). The deleted fragments were released by digestion with HindIII and inserted into the BamHI/HindIII sites of plasmid pUC-CAT. All deletions were sequenced across the cloning junctions in the double stranded plasmid DNA using the reverse primer (Biolabs) according to Chen and Seeburg (20). The alpha-actin-CAT hybrid contains the promoter of the chicken cardiac actin gene as detailed elsewhere (21). RSV-CAT has been generously provided by B. Howard. pRSV-ßGal was provided by T. Edlund (22).

Transfection of DNA and assay of CAT activity in cell extracts

Chicken myoblasts were transfected on 25 cm^2 flasks with double banded, supercoiled DNA and CAT activity was determined as described by Gorman (23). The calcium phosphate precipitate was added to the cells about 24 hrs after plating and remained

there for 20 min at room temperature and then for 6 hrs in the presence of medium at 37°C. For the CAT assay cells were scrapped off the plate 4 days after transfection when they had differentiated into contractile myocytes. The cells were washed in PBS and resuspended in 200 to 300 µl of 0.25 M Tris HCl pH 7.5. Cell extracts were prepared by three cycles of freezing and thawing with intermittent vortexing. In a 70 µl assay 20 % of total extract was used with 1 µCi of ^{14}C-chloramphenicol for 60 min. The test conditions were either standardized for percentage of total cell extract or for protein concentration in the assay. Protein content in cellular extracts was determined with the Biorad-Kit according to the procedure of the supplier. Both calibrations gave essentially the same results. Where indicated plasmid pRSV-βGal (5 µg) was cotransfected as internal standard. Beta-galactosidase activity was determined in 10 % of the total cellular extracts according to the procedure of An et al. (24). Thin layer plates were exposed on X-Ray films for 18 to 24 hrs.

S1 nuclease mapping

The LC-pUC-CAT transcripts were measured with the 450-bp Eco RI fragment cut from LC-pUC-CAT and labelled at the 5'end with (γ -^{32}P) ATP (5000 Ci/mol) and polynucleotide kinase (25). About 30.000 cpm (Cerenkov) of probe were precipitated with up to 200 µg of total RNA and hybridized at 42°C for 18 hrs in 80 % formamide, 40 mM Pipes pH 6.4, 0.4 M NaCl, 1 mM EDTA. The mixture was then diluted with 0.3 ml of buffer containing 0.3 M NaCl, 0.05 M Na-acetate pH 4.5, 4.5 mM Zn-sulfate and treated with nuclease S1 as described (26). The protected DNA fragments were analyzed on 7 M urea / 8 % polyacrylamide sequencing gels (18).

Preparation of RNA and Northern blot analysis

Total cellular RNA was extracted from tissue culture cells using the guanidinium /cesium chloride method (27). For Northern blot analysis the RNA was glyoxylated (28), separated on agarose gels and transferred onto nitrocellulose filters as described (29). Hybridization with nick translated cDNA probes for chicken LC_1/LC_3 (30) or LC 2-A (31), was performed in 50 % formamide, 5 x SSC (0.6 M NaCl, 0.06 M Na-citrate), 5 x Denhardt's solution (0.1 % each of Ficoll, bovine serum albumin and polyvinyl pyrrolidone), 0.05 M Na-phosphate pH 7.5 and 0.1 % SDS for 18 hrs at 42°C. Filters were washed at a final stringency of 0.1 x SSC, 0.1 % SDS at 65°C for 30 min.

RESULTS

Construction of MLC2-A promoter/CAT plasmids

The bacterial CAT gene, used as the indicator gene in all hybrids described here was supplied either in the pUC-CAT plasmid constructed in our laboratory or in the pEMBL-CAT plasmid, a derivative of pEMBL8 (17). As shown in figure 1A, various length fragments of the 5'-region of the MLC2-A gene were fused to CAT and the correct

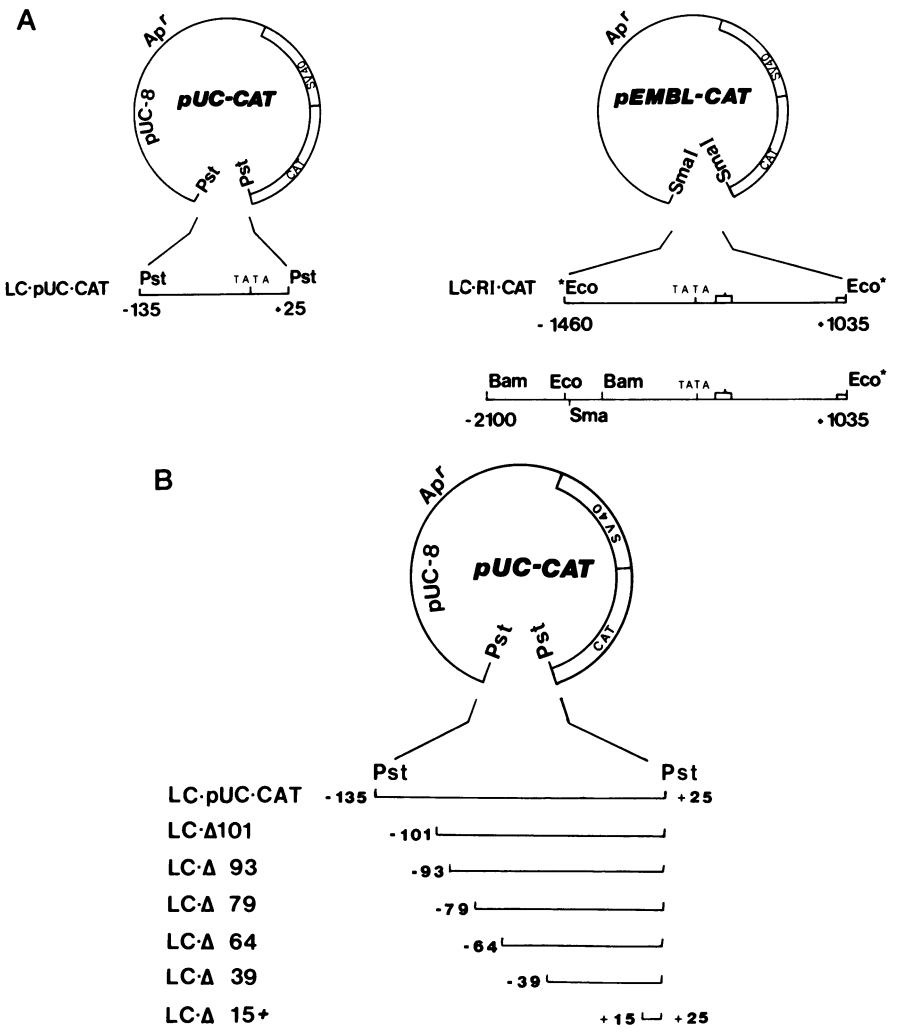

Fig. 1A: Construction of CAT-hybrid genes containing the chicken cardiac myosin light chain 2 (MLC2-A) promoter. Cloning vectors pUC-CAT and pEMBL-CAT carrying the bacterial CAT gene, the SV40 polyadenylation site (SV40) and the ampicillin resistance gene (Ap^r) were fused to fragments of the MLC2-A gene. These fragments were obtained by digestion with restriction endonucleases as indicated in the figure. The numbers relate to nucleotides upstream (-) or downstream (+) of the in vivo start site of transcription. Eco* indicates the fact that these sites of the original fragment were lost by the cloning procedure. The open boxes indicate exons. The putative promoter is designated as TATA element.

Fig. 1B: Construction of CAT-hybrid genes containing deletions of the MLC2-A upstream region in LC-pUC-CAT as detailed in Materials and Methods. The numbers indicate the extension of the MLC2-A elements in relation to the transcription start site.

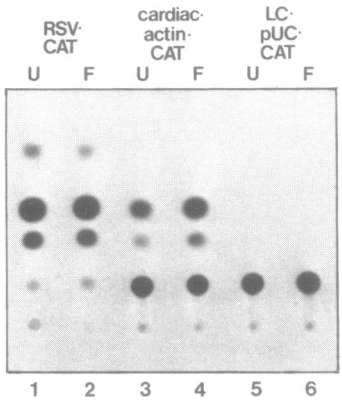

Fig. 2: Transient expression of CAT activity in C2C12 mouse muscle cells. Undifferentiated cultures ($1x10^6$ cells per 25 m^2 dish) were transfected 24 hrs after plating with 20 µg of RSV-CAT DNA or with 20 µg of CAT constructs under the control of chicken muscle gene promoters (cardiac actin; MLC2-A). CAT enzyme activity was determined in cell extracts 4 days after transfection either in fused myotubes (F) or in undifferentiated myoblasts (U). Differentiation was initiated 6 hrs after transfection by the addition of fusion medium (Blau et al., 1983). Cells were transfected with RSV-CAT DNA (lanes 1-2), cardiac actin-CAT (lanes 3-4) or LC-pUG-CAT (lanes 5-6). CM indicates chloramphenicol; AC-CM and 3 AC-CM stand for the acetylated forms of chloramphenicol.

orientation of the inserts was determined by sequencing both cloning junctions. In LC-pUC-CAT a 160-bp PstI fragment from positions -135 to +25 in relation to the cap site, was cloned into the PstI site of pUC-CAT. A series of deletion mutants containing decreasing length of 5'flanking sequences was generated from LC-pUC-CAT by exonuclease III digestion (see figure 1B) In LC-RI-CAT the MLC2-A promoter is contained in a 2500-bp Eco RI fragment from positions -1460 to 1035. This fragment also contains the complete first exon and intron plus 36 nucleotides from the second exon. It was fused into the SmaI site of pEMBL-CAT whereby a new translational start site was created. Plasmid 3.1-LC-CAT contains an extension of about 600 nucleotides of 5'-upstream sequences compared to LC-RI-CAT. The RSV-CAT plasmid (23), the beta actin-CAT plasmid constructed here, and the alpha-cardiac actin promoter CAT construct (21) were used as controls for the experiments described below.

Muscle specific expression of MLC2-A/CAT hybrid genes introduced into chicken myogenic culture cells.

The initial experiments to express the putative MLC2-A promoter were performed by stable transfections of various promoter constructs or the entire MLC2-A gene into the C2C12 mouse myoblast line (32, 33) or the L8 rat muscle cells (34). Both established rodent muscle lines were unable to sustain the activation of the MLC 2-A promoter in

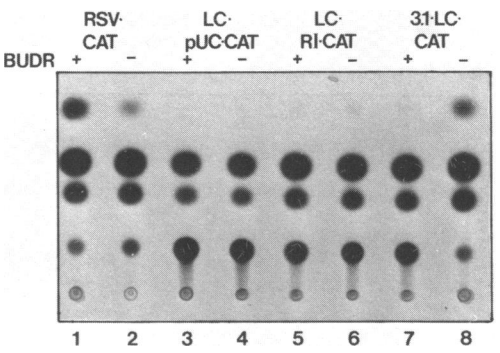

Fig. 3: Transient expression of CAT activity in primary cultures of chicken embryonic heart cells under the control of the MLC2-A promoter. Cardiocytes were grown either in the presence of 1.6×10^{-5} M BUdR (+) or in the absence of BUdR (-) and transfected with the indicated CAT-constructs 24 hrs after plating as described in Materials and Methods. CAT activity was determined 3 days later with 5 % (RSV-CAT) or 20 % of the cellular extracts of each dish.

unfused myoblasts or in fused myotubes, although control genes, such as the chicken ß-actin and the selectable marker gene conferring neomycin resistance were readily expressed in both cells (data not shown). Also, by transient transfection of LC promoter-CAT hybrids into C2C12 cells no CAT activity was detected whereas RSV-CAT or the chicken cardiac actin promoter showed high level expression (figure 2). Sequence analysis of the transfected LC-CAT constructs (LC-pUC-CAT and pEMBL-RI-CAT) indicated the correct insertion of the putative promoter element and showed no obvious destruction of the CAT reading frame. These results suggested that either essential elements of the transfected gene were missing from our constructs or the cellular background derived from rodent skeletal muscle was incompatible with the expression of the chicken cardiac myosin light chain 2 gene. This could be attributed to possible species specific cellular factors or to the fact that the cardiac gene can not be activated in skeletal muscle cells. In order to test for possible cellular requirements for the MLC2-A promoter activation, the described LC-CAT constructs were used for transient expression assays in primary cultures of chicken heart (15) or breast muscle cells (14). To prevent myogenic differentiation in control cultures, parallel dishes were grown in the presence of bromodeoxyuridine (BUdR), a thymidine analog that when incorporated into replicating DNA reversibly blocks the cellular differentiation of primary chicken myoblasts (35, 13). To introduce plasmid DNA. embryonic chicken myoblasts from breast or hearts were plated at a density of $1\text{-}2 \times 10^6$ cells per dish and allowed to grow for about 24 hrs; 20 micrograms of calcium phosphate precipitated DNA from hybrid constructs was transfected onto each dish and cells were harvested 3

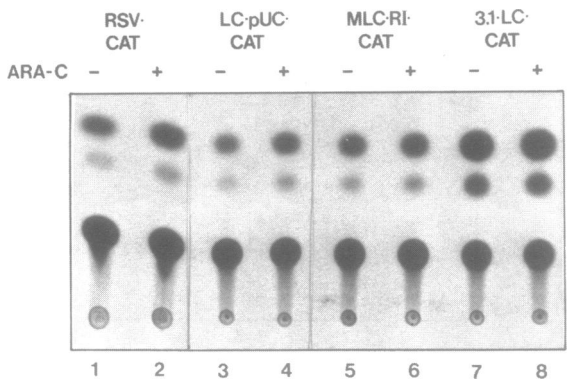

Fig. 4: Transient expression of CAT activity in primary heart cultures with (+) or without (-) the cytotoxic agent cytosine arabinoside (Ara-C). Cells were cultured and transfected as described in legend to fig. 3. Ara-C was added 6 hrs after transfection at a concentration of 100 µg per ml of medium.

or 4 days later when the majority had formed multinucleated myotubes or spontaneously beating cardiocytes. To evaluate promo ter activity of the chicken sequences joined to the CAT gene the enzymatic CAT activity was assayed and compared with RSV-CAT or cardiac actin-CAT in extracts from undifferentiated and differentiated cell cultures. As shown in figure 3, the various MLC-CAT plasmids when transfected into primary cultures of cardiac myoblasts directed high levels of chloramphenicol acetyltransferase activity in contrast to the results we had obtained in mouse and rat muscle cell lines. A slightly higher CAT activity was observed for the longer upstream sequences in pEMBL-RI-CAT and in 3.1-LC-CAT than for the 160-bp PstI promoter fragment in LC-pUC-CAT. The transcriptional activity appeared almost unaffected by the presence of BUdR indicating that the cardiocytes might not incorporate the thymidine analog and therefore continue to differentiate. To test whether BUdR had no effect on cardiocytes due to their general inability to replicate in culture, parallel dishes were grown in the absence or presence of cytosine arabinoside (ara-C). Ara-C is a cytotoxic inhibitor of DNA synthesis that leads to the elimination of dividing cells from cultures. Under these conditions one would expect diminished CAT signals from the MLC2-A constructs if premitotic cells have been transfected. As the results shown in figure 4 indicate, there is no reduction in the intensity of CAT signals with or without ara-C. This suggests that primary cardiocytes in culture do not undergo significant proliferation and are therefore transfected as postmitotic differentiated muscle cells. It also supports the interpretation that MLC2-A expression predominantly takes place in postmitotic heart muscle cells.

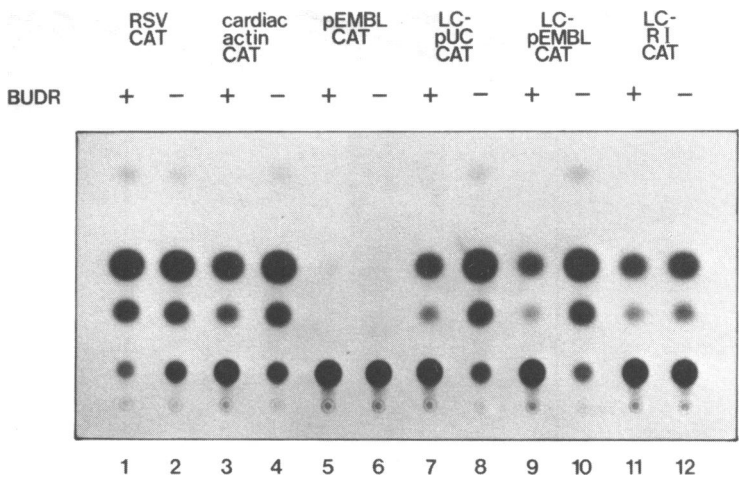

Fig. 5: Transient expression of CAT activity in primary cultures of chicken embryonic breast muscle cells under the control of the cardiac myosin light chain 2 promoter. The concentration of BUdR and the experimental procedures are given in the legend of fig. 3 and in Materials and Methods. pEMBL-CAT is the control without any eucaryotic promoter.

An even better indication for differentiation-dependent expression of the MLC2-A promoter was observed in transfection experiments in chicken primary breast muscle cells (figure 5). In these cells accumulation of CAT enzyme activity was clearly enhanced when cells had fused to form contractile myotubes (no BUdR). In the presence of BUdR, however, differentiation of dividing myoblasts was blocked and as a consequence the LC promoter dependent CAT activity was low. About 130 nucleotides of DNA upstream of the transcription start site appeared to be sufficient for fusion enhanced expression of the MLC2-A promoter (see LC-pUC-CAT or LC-pEMBL-CAT in figure 5), since larger upstream sequences (up to 2.1 kb in 3.1-LC-CAT) and the inclusion of the complete first intron did not affect MLC2-A-CAT activity. RSV-CAT and cardiac actin CAT used as references were also highly active in breast muscle cells but as expected the activity of RSV-CAT was not diminished by BUdR whereas cardiac-actin CAT exhibited measurable up-regulation of the promoter activity during myotube formation. These same results were consistently obtained in a minimum of 6 separate experiments performed with at least 2 independently prepared plasmid preparations for each construct.

The muscle cell specific expression of LC promoter dependent CAT activity was furthermore demonstrated by transfecting LC-CAT hybrid plasmids into chicken primary fibroblast cultures. As shown in figure 6, the CAT gene driven by the LC2

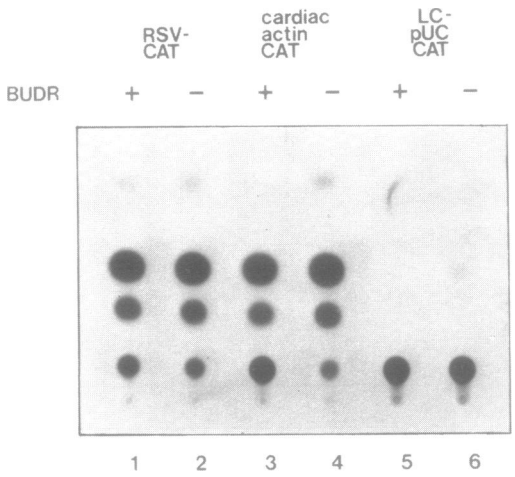

Fig. 6: Transient expression of MLC-promoter controlled CAT activity in primary cultures of chicken embryonic skin fibroblasts. Results are shown for RSV-CAT (lanes 1 + 2), cardiac actin-CAT (lanes 3 + 4) and LC-pUC-CAT (lanes 5 + 6). No CAT activity was found for LC-RI-CAT and 3.1.-LC-CAT constructs (data not shown).

promoter was inactive in fibroblasts regardless whether or not BUdR was present. The same result was obtained for all other described LC-CAT constructs (data not shown). In contrast, RSV-CAT and cardiac actin-CAT used as control constructs were efficiently transcribed in these nonmuscle cells. An extremely faint signal (less than 1 percent of RSV-CAT) was sometimes observed. This could either be due to some contaminating muscle cells in the primary skin fibroblasts or to the known basal activity of even promoterless CAT constructs in some cells. The vast difference in CAT activities, however, clearly indicates a specific prevalence of the MLC2-A promoter for muscle cells.

Cis-regulatory sequence elements required for promoter activity in muscle cells

In order to delineate the essentially required sequences for the promoter activity in muscle cells, a series of deletion mutants were made from LC-pUC-CAT DNA (figure 1B). The CAT activity of these constructs was tested in chicken primary heart cells and also in breast muscle cultures with or without BUdR. As an internal control for the efficiency of transfection pRSV-ßGal plasmid (22) was cotransfected and actual CAT conversion was expressed as a ratio to ß-galactosidase activity. At the concentrations used in these experiments no competition has been observed between the LC promoter of the reporter CAT gene and the pRSV-ß-Gal promoter. As shown in figure 7 the MLC 2-A promoter activity was maintained in heart cells (fig. 7b) as well as in skeletal muscle cells (fig. 7a) in 5' deletion mutants down to 64 nucleotides upstream of the

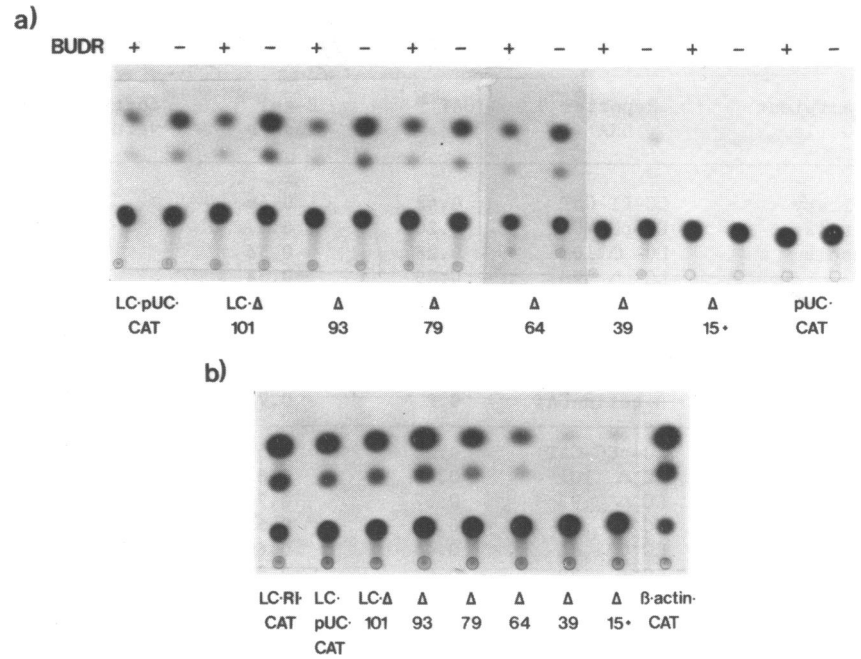

Fig. 7: Transient expression of LC-CAT deletion mutants in primary cultures of embryonic breast muscle cells cultured with or without BUdR (7a) and embryonic heart cells (7b). Experimental conditions were as described in Materials and Methods. For each construct 10 µg of DNA were cotransfected with 10 µg of pRSV-ßGal. The data shown were obtained with 10 % of extract for heart cells and 5 % of extract for breast muscle cells. For each reaction the acetylated products and the unreacted chloramphenicol were cut from the thin layer plate and counted in a scintillation counter (results are shown in table 1).

transcription start site. A precipitous drop in activity was observed when shorter promoter fragments were used. No major difference of promoter activity, however, was observed for the deletions LC 101 to LC 64. As shown in fig. 7a and table 1, several fold higher CAT activity was measured in myotubes (no BUdR) compared to predominantly myoblasts (plus BUdR). None of the constructs described in this experiment showed any CAT activity above background levels in chicken embryonic skin fibroblasts. The data suggest, that for differentiation dependent and modulated expression of the MLC 2-A promoter in myogenic culture cells sequences close to the TATA consensus element seem to be required and possibly sufficient.

Evidence for the correct initiation of transcription in transfected LC-CAT constructs

To ensure that LC-CAT transcripts were initiated at the correct start site in transfected cells we utilized an S1 protection assay and compared the results to the previously determined cap site of the MLC2-A gene in vivo (3). When the 450-bp Eco RI

Table 1
CAT Activity of 5'Deletion Mutants of the LC2-A Promoter[e)]

Recipient Cells	Reporter DNA [a]	CAT assays [b]	ß-gal assays [c]	corrected values for CAT [d]
Primary cardiac muscle	LC-RI-CAT	0.48	0.66	0.72
	LC-pUC-CAT	0.20	0.57	0.35
	LC-Δ 101	0.28	0.56	0.50
	LC-Δ 93	0.29	0.54	0.53
	LC-Δ 79	0.38	0.37	1.00
	LC-Δ 64	0.18	0.32	0.56
	LC-Δ 39	0.05	0.60	-
	LC-Δ 15+	0.05	0.24	-
	ß-actin-CAT	0.8	0.71	1.1
Primary breast myotubes (-BudR)	LC-pUC-CAT	0.23	0.55	0.42
	LC-Δ 101	0.40	0.62	0.64
	LC-Δ 93	0.67	0.76	0.88
	LC-Δ 79	0.36	0.53	0.67
	LC-Δ 64	0.44	0.60	0.73
	LC-Δ 39	0.01	0.52	-
	LC-Δ 15+	0.01	0.46	-
	ß-actin-CAT	0.6	0.61	0.98
Primary breast myoblasts (+BudR)	LC-pUC-CAT	0.11	0.61	0.18
	LC-Δ 101	0.16	0.77	0.20
	LC-Δ 93	0.18	0.77	0.23
	LC-Δ 79	0.12	0.61	0.2
	LC-Δ 64	0.10	0.56	0.18
	LC-Δ 39	0.005	0.84	-
	LC-Δ 15+	0.005	0.65	-
	ß-actin-CAT	0.65	0.7	0.9

a) Transfections were performed with 15 ug of reporter DNA plus 5 ug of pRSV-ßgal plasmid.

b) Numbers represent ratio of cpm in acetylated derivatives to total cpm on the TLC plate. Assays were performed with 10% of cellular extract for cardiac muscle and 5% of extract for breast muscle.

c) ß-gal assays were carried out with 10% of cell extracts as described under Materials and Methods. Values have been corrected for extracts of mock transfected cells.

d) Corrected values for CAT-activity were derived by dividing the CAT values by the ß-gal values.

e) Values represent means of at least 5 experiments.

fragment from LC-pUC-CAT was radiocatively labelled at its 5'end and hybridized to RNA from cultured breast muscle cells, transfected with LC-pUC-CAT, the expected fragment of 290 nucleotides was protected upon challenge with S1 nuclease (figure 8). RSV-CAT or ß-actin pomoter-CAT constructs transfected as control also exhibited the

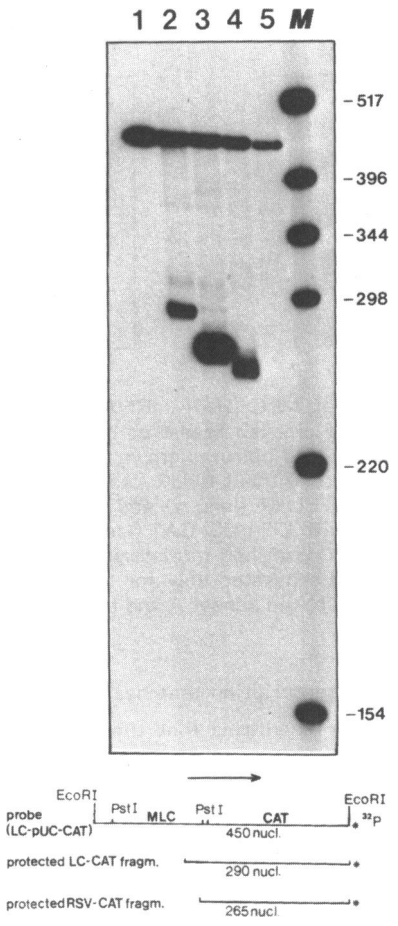

Fig. 8: S1 analysis of the transcriptional start site for the MLC2-A promoter CAT fusion plasmids. The probe was generated from the LC-pUC-CAT plasmid by digestion with Eco RI and 5'end-labelling with ^{32}P- ATP and T4 polynucleotide kinase (lane 1 untreated probe). The probe was hybridized with 20 - 100 µg of RNA from fused breast muscle cultures transfected with LC-pUC-CAT (lane 2), RSV-CAT (lane 3), chicken ß-actin CAT (lane 4) or untransfected breast muscle cells (lane 5). Digestion with S1 nuclease and gel electrophoresis were performed as described in Materials and Methods. As shown in the figure, the correct start sites predict a 290 nucleotide fragment for LC-CAT, 265 nucleotide fragment for RSV-CAT and an approximately 260 nucleotide fragment for ß-actin-CAT.

correct size fragments of 265 and 280 nucleotides, respectively. We observed a slight microheterogeneity at the start site, a phenomenon that has also been described for the LC_1 and LC_3 genes of skeletal muscle in chicken (30), mouse (36) and rat (37). Our result indicates that in cultured cells transfected with the LC-CAT hybrid gene the

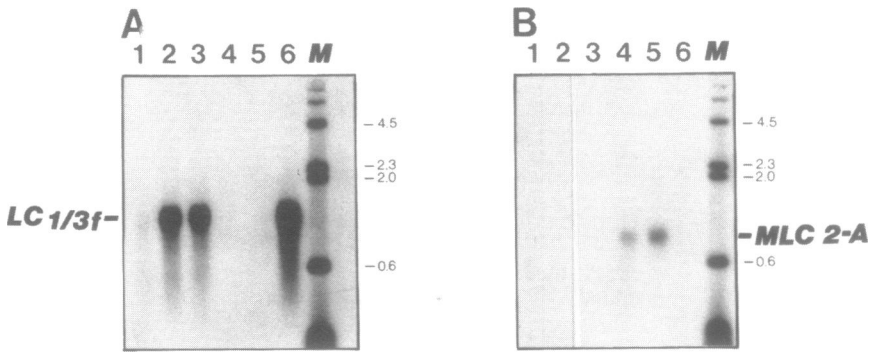

Fig. 9: Northern blot analysis of MLC 1f/3f mRNA (pannel A) or MLC2-A mRNA (pannel B) in primary cultures of chicken breast or heart muscle. Ten micrograms of total RNA from 4 day old breast cell cultures grown in the presence of BUdR (lane 1), from 3 or 4 day old breast cultures without BUdR (lanes 2 and 3, respectively), from 4 day old heart cell cultures with BUdR (lane 4) and without BUdR (lane 5) and from breast cell cultures transfected with LC-pUC-CAT (lane 6) were denatured, electrophoretically analyzed and blotted as described in Materials and Methods. Both blots were generated from the same gel and separated only for the hybridization with cloned and radioactively labelled MLC1/3 cDNA in pannel A and the cloned cDNA probe for MLC2-A in pannel B.

identical start site is used for transcription that has been shown in normal embryonic chicken hearts (3). It furthermore confirms that the same promoter element that is active in vivo only in myocardial cells is capable of exerting its activity also in skeletal muscle cells.

The endogenous MLC2-A gene is not expressed in primary skeletal muscle cultures

The observation that the transfected MLC2-A promoter is active in cultured skeletal muscle cells leads to the possibility that the endogenous MLC2-A gene might also be expressed under the culturing conditions. To answer this question RNA was isolated from breast and heart muscle cells cultured with or without BUdR and also from cells that had been transfected with LC-pUC-CAT. The samples were analyzed in duplicates on northern blots and hybridized either with the chicken LC_1/LC_3 cDNA probe or with the cardiac myosin LC2-A cDNA. As presented in figure 9, breast muscle myotubes synthesized only the expected LC_1/LC_3 mRNA whereas cultured cardiocytes only transcribed the MLC2-A mRNA. There was no promiscous expression of either light chain species in the analyzed cultures, even when cells had been compromised by the calcium phosphate precipitate. There was, however, a significant difference in the effect of BUdR on skeletal and cardiac cells. Whereas the transcription of LC_1/LC_3 mRNA was almost completely suppressed by BUdR, the expression of MLC2-A mRNA in heart cells remained almost unaffected. This supports our earlier observation that BUdR has no significant effect on cardiocytes.

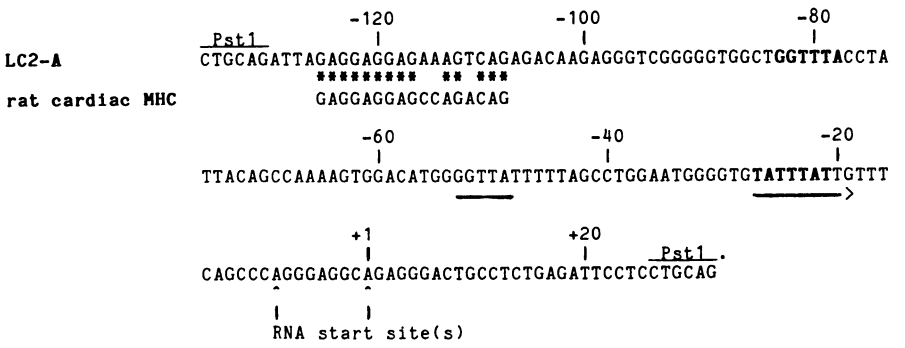

Fig. 10: The nucleotide sequence of the chicken MLC2-A promoter fragment. Promoter consensus motifs are printed in fat letters. Note that the putative CCAAAT-box at -80 is located on the opposite strand. Another putative "CCAAT" motif on the noncoding strand and the "TATA"-box are underlined. 17 nucleotides of the MLC2-A gene show a 83% homology to a sequence element located in the rat cardiac myosin heavy chain (MHC) gene at a similar position.

The proximal promoter sequence of the MLC2-A gene

A summary of potentially important sequence elements within the small PstI-promoter fragment of the MLC2-A gene is shown in figure 10. A computer-search for homologies with other muscle genes under reasonably selective conditions (more than 70 % overall homology) revealed a sequence element of 17 nucleotides with a 83 % homology to the cardiac myosin heavy chain gene of rat. This sequence element is located about 90 nucleotides upstream of the "TATA"-box in chicken and at approximately the same position in the rat gene. By deletion analysis, however, we were unable to demonstrate a functional significance of this sequence for the promoter activity under our assay conditions. Likewise the deletion of the potential CCAAT-box at position -80 (on the noncoding strand) or the potential CArG box (CCAAAAG) did not appreciably change the promoter function. In contrast, a "CCAAT" motif located on the noncoding strand around the position -50 leads, when deleted, to a drastic reduction of the transcriptional activity.

DISCUSSION

The tissue- and developmental stage-specific expression of various isoforms of muscle proteins from their respective genes is precisely controlled during myogenesis. Since muscle cells in culture can be readily triggered to differentiate from proliferating myoblasts to fused myotubes they are suitable to study the mechanisms responsible for gene activation and isoform switching that takes place during that process. In the present work we take advantage of this system and demonstrate that recombinant plasmids containing 5'-flanking segments of the chicken MLC2-A gene linked to the

bacterial CAT gene are transcribed at high levels in chicken primary muscle cultures, but not in nonmuscle cells or established rodent myoblast lines. This cell type-specific expression is shown for hybrids containing up to 2100 nucleotides of sequence upstream of the RNA start site as well as the complete first intron of the gene. Removal of large segments of upstream sequences and the first intron does not greatly influence the level of CAT expression. This indicates that these sequences might not be essential for the promoter activity itself nor for its enhanced expression. The smallest hybrid construct that was sufficient to maintain high levels of CAT expression in a muscle specific manner contained only 64 nucleotides 5' to the start site of transcription. It is interesting to note that this sequence does neither include the putative CCAAAT element (in the MLC2-A gene located on the noncoding strand) at position -80 nor the CArG-box like sequence CCAAAAG that was postulated to confer tissue-specificity to the actin and other muscle promoters (11, 38)

In contrast to our results, Zarraga et al. (7) described activity of the MLC2-A promoter in the quail fibroblast cell line QT35 and in COS cells. In their constructs the promoter is contained in a 1.6 kb Hind III fragment that also supplies the AUG translational start codon of MLC2-A and therefore needs to be fused in the reading frame of the CAT gene. In an attempt to reproduce the expression of the MLC 2-A promoter in a nonmuscle background, as suggested by these authors, we transfected our LC-CAT constructs as well as those constructed in our laboratory according to their procedure in QT 35 or in COS cells. Although we were unable to obtain specific expression in either cell, we did observe unspecific CAT enzyme activity in QT 35 cells and also in a quail cell line which was transformed by a temperature senitive RSV-mutant (unpublished observations). This activity, however, was also obtained with pSV-O-CAT, a promoter-less CAT plasmid, and in quail muscle cells regardless whether or not they were differentiated. We therefore like to suggest that transformed quail cells seem to be very permissive for unspecific CAT expression and can not be used to prove muscle specific activation of a gene promoter.

It has been extensively documented that chicken myoblasts and other cell types (34, 13) grown in the presence of BUdR are completely, but reversibly blocked in their capacity to form the functional muscle phenotype. To prevent spontaneous differentiation in primary cultures and in order to show the differentiation dependent up-modulation of the muscle promoter activity we took advantage of this observation. As our results clearly demonstrate, the LC2-A promoter activity is significantly suppressed in myoblasts grown in the presence of BUdR whereas the promoters of nonmuscle genes like the LTR of Rous sarcoma virus or the chicken ß-actin promoter (data not shown) appeared to be unaffected by the BUdR treatment. This observation is in agreement with the fact that cells are capable of undiminished growth for several generations in

concentrations of BUdR that completely inhibit myogenesis but obviously do not prevent functions of cellular housekeeping genes. The mechanisms by which BUdR prevents the formation of CAT mRNA under the control of the MLC2-A promoter is not fully understood. In our studies there is no opportunity for replication or integration of the LC-CAT plasmids under the conditions of transient expression. It is clear, then, that BUdR does not have to be incorporated directly into the promoter of the muscle specific gene, to exert its effect. The effect of BUdR could therefore be mediated by a transacting factor(s). There is now ample evidence for the involvement of transacting factors or activators in muscle (39) as well as in other tissues. BUdR may somehow suppress the synthesis of a putative myogenic activator.

Most of the sequence contained in the largest construct, 3.1.-LC-CAT, did not seem to be essential for muscle specific and regulated expression of the MLC2-A mRNA. However, the cardiac muscle subtype (cardiac versus skeletal) specifity may require elements which are either not present or exert no control under our assay conditions. It is conceivable that, while about 65 nucleotides around the promoter are sufficient to confer muscle specific transcriptional activity, additional DNA elements located further upstream or in the gene body or in the 3'flanking region are required for cardiac specific expression. Alternatively, other factors which cannot be studied in transient expression, such as conformational changes of chromatin structure or the state of methylation may influence gene activity. We have previously reported our findings on tissue-specific DNAse I hypersensitive sites and hypomethylation of the MLC2-A gene in heart which support this idea (3).

ACKNOWLEDGEMENTS

We wish to thank Drs. B. Winter and W. Quitschke for valuable help in the initial transfection experiments of primary cultures. We also acknowledge the technical assistance of I. Humburg and K. Söhren and the secreterial work of Chr. Mink and B. Everding. This investigation was supported by the Deutsche Forschungsgemeinschaft by a grant to H.H.A.

*To whom correspondence should be addressed

REFERENCES

1. Daubas, P., Caput, D., Buckingham, M., Gros, F. (1981), A comparison between the synthesis of contractile proteins and the accumulation of their translatable mRNAs during calf myoblast differentiation, Dev.Biol. 84: 133-143
2. Caravatti, M., Minty, A., Robert, B., Montarras, D., Weydert, A., Cohen, A., Daubas, P., Buckingham, M. (1982), Regulation of muscle gene expression. The accumulation of mRNAs coding for muscle specific proteins during myogenesis in a mouse cell line, J.Mol.Biol. 160: 59-76

3. Winter, B., and Arnold, H.H. (1987), Tissue-specific DNAse I-hypersensitive sites and hypomethylation in the chicken cardiac myosin light chain gene (L2-A), J.Biol.Chem. 262, 13750-13757
4. Buckingham, M.E. (1985), Actin and myosin multigene families: Their expression during the formation of skeletal muscle, in: Campell, P.H., Marshall, R.D. (eds.) "Essays in Biochemistry", New York, Academic Press, Vol. 20, pp. 77-109
5. Winter, B., Klapthor, H., Wiebauer, K., Delius, H., Arnold, H.H. (1985), Isolation and characterization of the chicken cardiac myosin light chain (L2-A) gene, J.Biol.Chem. 260: 4478-4483
6. Lohse, P., Winter, B., Mouly, V., Fiszman, M.Y., Arnold, H.H. (1987), Evidence for distinct phosphorylatable myosin light chains in avian heart and slow skeletal muscle, Dev. Biol., in press.
7. Zarraga, A.M., Danishefsky, K., Desphande, A., Nicholson, D., Mendola, C., Siddiqui, M.A.Q. (1986), Characterization of 5'-flanking region of heart myosin light chain 2A gene, J.Biol.Chem. 261: 13852-13860
8. Walker, M.D., Edlund, T., Boulet, A.M., Rutter, W.J. (1983), Cell-specific ex pression controlled by the 5'-flanking region of insulin and chymotrypsin genes, Nature 306: 557-561
9. Ott, M.O., Sperling, L., Hermobel, P., Yaniv, M., and Weiss, M.C. (1984), Tissue-specific expression is confered by a sequence from the 5' end of the rat albumin gene, EMBO J. 3: 2505-2510
10. Melloul, D.D., Aloni, B., Calvo, J., Yaffe, D., Nudel, U. (1984), Developmentally regulated expression of chimeric genes containing muscle actin DNA sequences in transfected myogenic cells, EMBO J. 3: 983-990
11. Minty, A. and Kedes, L. (1986), Upstream regions of the human cardiac actin gene that modulates its transcription in muscle cells: presence of an evolutionarily-conserved repeated motif, Mol.Cell.Biol.6: 2137-2145
12. Bergsma, D., Grichnik, J.M., Lani, M., Gossett, A., Schwartz, R. (1986), Delimitation and characterization of cis-acting DNA sequen ces required for the regulated expression and transcriptional control of the chicken skeletal -actin gene, Mol.Cell.Biol. 6: 2462-2475
13. O'Neill, M.C. and Stockdale, F.E. (1974), 5-BUdR inhibition of differentiation. Kinetics of inhibition and reversal in myoblasts, Dev.Biol. 37: 117-132
14. Paterson, B.M., and Strohman, R.C. (1972), Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle, Dev.Biol. 29: 113-138
15. Clark, Jr., W.A. and Fischman, D.A. (1983), Analysis of population cytokinetics of chick myocardial cells in tissue culture, Dev.Biol. 97: 1-9
16. Seiler-Tuyns, A., Eldrige, J.D., Paterson, B.M. (1984), Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells, Proc.Natl.Acad.Sci. USA 81: 2980-2984
17. Dente, L., Sollazzo, M., Baldari, G., Cesareni, G., Cortese, R. (1985), The pEMBL family of single stranded vectors, in Glover, D.M. (ed.): "DNA Cloning", Vol.I, pp. 101-107, Washington DC., IRL Press.
18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977), Proc.Natl.Acad.Sci. USA 74, 5463-5467
19. Henikoff, S. (1984), Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing, Gene 28: 351-359
20. Chen, E.J. and Seeburg, P.U. (1985), Supercoil sequencing: a fast and simple method for sequencing plasmid DNA, DNA 4, 165-170
21. Quitschke, W., Eldrige, J.D., Paterson, B.M., in: Mechanisms of Control of Gene Expression, UCLA Symposion on Molecular and Cellular Biology, New Series, Vol.67, eds. B. Cullen, L.P. Gage, M.A.Q. Siddiqui, A.M. Skalka and H. Weissbach, Alan R. Liss, Inc., New York, N.Y. (1987)
22. Edlund, T., Walker, M.D., Barr, P.J. and Rutter, W.J. (1985), Cell-specific expression of the rat insulin gene: Evidence for role of 2 distinct 5'-flanking elements, Science 230, 912-916

23. Gorman, C. (1985), High efficiency gene transfer into mammalian cells, In Glover, D.M. (ed.): "DNA Cloning", Vol. II, pp. 143-190, Washington DC., IRL Press.
24. An, G., Hikada, K. and Siminovitch, L. (1982), Mol.Cell.Biol.2: 1628-1632
25. Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p.123
26. Berk, A.J. and Sharp, P.A. (1977), Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-diges ted hybrids, Cell 12: 721-732
27. Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W.J., Goodman, H.M. (1977), Rat insulin genes: Construction of plasmids containing the coding sequences, Science 196: 1313-1319
28. McMaster, G.K. and Carmichael, G.G. (1977), Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acidine-orange Proc.Natl.Acad.Sci.USA 74: 4835-4838
29. Thomas, P.S. (1980), Hybridization of denatured RNA and small DNA fragments transfered on nitrocellulose, Proc.Natl.Acad.Sci. USA 77: 5201-5205
30. Billeter, R., Quitschke, W., Paterson, B.M. (1988), BUdR suppresses the trans-activation of the chicken myosin light chain 1 and 3 gene promoter elements in primary cultures of embryonic chick muscle, Mol.Cell.Biol. in press.
31. Arnold, H.H., Krauskopf, M., Siddiqui, M.A.Q. (1983), Nucleic Acids Res.11: 1123-1131
32. Blau, H.M., Chiu, C.-P. and Webster, C. (1983), Cytoplasmic activa tion of human nuclear genes in stable heterokaryons, Cell 32: 1171-1180
33. Yaffe, D., and Saxel, O. (1977 a), serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle, Nature 270: 725-727
34. Yaffe, D., and Saxel, O. (1977 b), A myogenic cell line with altered serum requirements for differentiation, Differentiation 7: 159-166
35. Bischoff, R. and Holtzer, H. (1970), Inhibition of myoblast fusion after one round of DNA synthesis in 5-bromodeoxyuridine, J.Cell.Biol.44: 134-150
36. Daubas, P., Robert, B., Garner, J., Buckingham, M. (1985), A comparison between mammalian and avian fast skeletal muscle alkali myosin light chain genes, Nucleic Acids Res. 13: 4623-4643
37. Strehler, E.E., Periasamy, M., Strehler-Page, M.-A., Nadal-Ginard, B. (1985), Myosin light chain 1 and 3 gene has two structurally distinct and differentially regulated promoters evolving at different rates, Mol.Cell.Biol. 5: 3168-3182
38. Miwa, T., Boxer, L.M., Kedes, L. (1987), CArG boxes in the human cardiac -actin gene are core binding sites for positive transacting regulatory factors, Proc.Natl. Acad.Sci.USA 84, 6702-6706
39. Blau, H.M., Pavelath, G.K., Hardeman, E.C., Chiu, C.-P., Silberstein, L., Webster, S.G., Miller, S.C., Webster, C. (1985), Plasticity of the differentiated state, Science 230: 758-766