MOLECULAR AND CELLULAR BIOLOGY, Jan. 1988, p. 62–70 Vol. 8, No. 1
0270-7306/88/010062-09$02.00/0
Copyright © 1988, American Society for Microbiology

The Muscle Creatine Kinase Gene Is Regulated by Multiple Upstream Elements, Including a Muscle-Specific Enhancer

JAMES B. JAYNES,† JANE E. JOHNSON, JEAN N. BUSKIN, CYNDY L. GARTSIDE, AND STEPHEN D. HAUSCHKA*

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Received 12 June 1987/Accepted 24 September 1987

Muscle creatine kinase (MCK) is induced to high levels during skeletal muscle differentiation. We have examined the upstream regulatory elements of the mouse MCK gene which specify its activation during myogenesis in culture. Fusion genes containing up to 3,300 nucleotides (nt) of MCK 5′ flanking DNA in various positions and orientations relative to the bacterial chloramphenicol acetyltransferase (CAT) structural gene were transfected into cultured cells. Transient expression of CAT was compared between proliferating and differentiated MM14 mouse myoblasts and with nonmyogenic mouse L cells. The major effector of high-level expression was found to have the properties of a transcriptional enhancer. This element, located between 1,050 and 1,256 nt upstream of the transcription start site, was also found to have a major influence on the tissue and differentiation specificity of MCK expression; it activated either the MCK promoter or heterologous promoters only in differentiated muscle cells. Comparisons of viral and cellular enhancer sequences with the MCK enhancer revealed some similarities to essential regions of the simian virus 40 enhancer as well as to a region of the immunoglobulin heavy-chain enhancer, which has been implicated in tissue-specific protein binding. Even in the absence of the enhancer, low-level expression from a 776-nt MCK promoter retained differentiation specificity. In addition to positive regulatory elements, our data provide some evidence for negative regulatory elements with activity in myoblasts. These may contribute to the cell type and differentiation specificity of MCK expression.

The differentiation of skeletal muscle in culture provides a model system for the study of developmental regulatory processes at the cellular level. The earliest phase of muscle cell differentiation, the transition from pluripotential mesodermal stem cells to myoblasts, can be studied using the 10T1/2 cell line (33, 35, 67), and the appearance of distinct myoblast types during embryogenesis can be monitored and analyzed using primary and subclonal populations of limb bud cells (58, 59). In later phases of myogenesis, the extracellular signals and ensuing intracellular events which control the transition from proliferating myoblasts to differentiated myocytes can be studied in various primary cultures and permanent myogenic cell lines (11a, 11b, 21, 44, 46, 47). Since a major transition in the pattern of protein synthesis occurs during myogenic terminal differentiation (6), this system can be used to investigate the mechanisms of coordinate gene expression through comparisons of the regulation of different muscle-specific genes (8, 13). Eventually, the understanding of a broad range of cellular processes in various myogenic cell culture systems should allow the formulation of a coherent model of muscle development.

Several muscle-specific genes are now known to be activated transcriptionally during myogenesis (21, 28, 32, 33, 39, 44, 47), and transcriptional regulation has been inferred for others from the accumulation of specific mRNAs (7, 13, 62). The *cis*-acting regulatory regions required for transcriptional induction have been examined for the chicken skeletal actin (3), quail troponin I (33, 33a), rat skeletal actin (40), human cardiac actin (45), and chicken acetylcholine receptor α-subunit (32) genes.

We have previously reported that the muscle creatine kinase (MCK) gene is transcriptionally activated during the differentiation of cultured MM14 mouse myoblasts and that such transcriptional activation could be conferred by 3,300 nucleotides (nt) of MCK 5′ flanking DNA (28; J. E. Johnson, C. L. Gartside, J. B. Jaynes, and S. D. Hauschka, submitted for publication). In this study we examine regulatory elements within the 5′ flanking region, and we have found an element located about 1,100 nt upstream of the transcription start site which has the properties of a muscle-specific enhancer. The MCK enhancer shares with other enhancers the ability to activate transcription in a position- and orientation-independent manner. Such elements have been found in many viral and cellular genes in various positions with respect to the transcription start site (1, 50, 54, 61) and are distinguishable from basal promoter elements, which are required in closer proximity to the site of transcription initiation (31, 71). Evidence has been presented for both positive (27, 30, 51) and negative (4, 57, 72) regulation of enhancer function. A complex, and not yet understood, interaction between enhancer, promoter, and *trans*-acting factors is involved in determining transcription levels (22, 29, 36, 53, 55, 66, 68, 70). Some enhancers have been implicated in the regulation of cell type-specific transcription (25) in the pancreas (23, 49, 65), the immune system (1, 16, 52), and the liver (22a), and a recent study with skeletal muscle has identified an enhancer element in intron 1 of the quail troponin I gene (33a).

MATERIALS AND METHODS

MM14 mouse myoblasts were cultured as described previously (28, 38). Exponentially growing cells were transfected essentially as described (10) at 300,000 to 600,000 cells per 100-mm dish, using 10 μg of plasmid DNA which included either 2 or 3 μg of a reference plasmid (RSV-lacZ; W. Albert, unpublished data). Briefly, calcium phosphate-

* Corresponding author.
† Present address: Department of Biochemistry, University of California Medical Center, San Francisco, CA 94143.

DNA precipitates were prepared (28) and applied to proliferating myoblast cultures. At 4 h later the cultures were glycerol shocked for 2.5 min and then either refed with proliferation medium, which included purified fibroblast growth factor, or switched to differentiation medium (without fibroblast growth factor) (37). Cells were harvested 24 h after glycerol shock and assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase as previously described (28), except that cells were disrupted either by sonication or by freeze-thaw (three times in dry ice-ethanol). Protein concentrations were determined by the method of Bradford (5).

L cells were cultured in Dulbecco modified Eagle medium–10% calf serum and were transfected essentially as described above for MM14 cells, except that glycerol shock was for 4 min and cultures were harvested 48 h after transfection.

Plasmid p118CAT (see Fig. 2, plasmid 4) was constructed by inserting the CAT gene-containing *Hin*dIII-*Bam*HI fragment from pRSVcat (19) into the *Bam*HI polylinker site of pUC118 (obtained from J. Vieira, J. Messing, and J. Norrander, Department of Biochemistry, University of Minnesota) such that the M13 *ori*-sequencing primer region was upstream. Plasmid −3300MCKCAT (see Fig. 2, plasmid 1) was made from p118CAT by inserting a mouse genomic MCK fragment (28) from nt −3300 to +7 (relative to the transcription start site) into the *Xba*I site immediately upstream of the CAT structural gene.

Deletions were made in −3300MCKCAT by digestion with *Pst*I (in the polylinker) and *Sal*I (at nt −2800) followed by treatment with exonuclease III. Following treatment with S1 nuclease and Klenow polymerase, the resulting blunt-ended plasmids were ligated and used to transform competent *Escherichia coli* cells (24). This gave unidirectional 5′ deletions and resulted in the deletion series shown in Fig. 1 (including plasmids 2 and 3 in Fig. 2), except −80MCKCAT (see Fig. 2, plasmid 5), which was derived from −3300 MCKCAT by digestion with *Sal*I (in the polylinker) and *Apa*I (at nt −80) and then blunt ending and ligation to delete MCK sequences upstream of nt −80 (28), and −776MCKCAT (see Fig. 2, plasmid 10), which was constructed by ligating the MCK genomic fragment from nt −776 to +7 into the *Xba*I site of p118CAT (upstream of the CAT gene). Plasmids en1(+)−80MCKCAT and en1(−)−80MCKCAT (see Fig. 2, plasmids 6 and 7, respectively) contain the MCK *Bgl*II-BamHI fragment (en1) from nt −1748 to −1050 in either orientation, (+) or (−), blunt-end ligated into the *Sal*I site of −80MCKCAT. Plasmids −776MCKCATen1(+) and −776MCKCATen1(−) (see Fig. 2, plasmids 11 and 12, respectively) contain the same MCK fragment ligated into the *Bam*HI site of −776MCKCAT. −80MCKCATen2(+) and −80MCKCATen2(−) (see Fig. 2, plasmids 8 and 9, respectively) contain the *Hin*dIII-*Bam*HI fragment (en2) from deletion plasmid 3 in Fig. 2 (MCK −1256 to −1050) similarly ligated into the *Bam*HI site of −80MCKCAT, while −776MCKCATen2(+) and −776MCKCATen2(−) (see Fig. 2, plasmids 13 and 14, respectively) contain the same fragment ligated into the *Bam*HI site of −776MCKCAT. Plasmids en1(+)TKCAT and en1(−)TKCAT (see Fig. 4, plasmids 16 and 17, respectively) and en2(+)TKCAT and en2(−)TKCAT (see Fig. 4, plasmids 18 and 19, respectively) contain the same MCK fragments ligated into the *Bam*HI site of TKCAT (43). SV40-TKCAT (see Fig. 4, plasmid 20) has the simian virus (SV40) enhancer-containing *Acc*I-*Sph*I fragment from SV_2CAT (20), blunt-end ligated into the same TKCAT upstream *Bam*HI site. SV_1CAT (see Fig. 4, plasmid 21) was a gift from Fayth Yoshimura and Tom Hollon and was derived from SV_2CAT by recloning the *Sph*I-*Bam*HI fragment (with the SV40 early promoter and CAT gene) into *Sph*I-*Hin*dIII-cut pUC18. en1(+)SV_1CAT and en1(−)SV_1CAT (see Fig. 4, plasmids 22 and 23, respectively) and en2(+)SV_1CAT and en2(−)SV_1CAT (see Fig. 4, plasmids 24 and 25, respectively) were derived from SV_1CAT by cloning the previously described MCK fragments into the (upstream) *Bam*HI site. Plasmid 26 in Fig. 4 is SV_2CAT.

RNA was isolated by the guanidine thiocyanate method (9). S1 analysis of MCK-CAT fusion gene transcripts involved the use of a probe, prepared as previously described (28), which was labeled at an *Eco*RI site within the CAT structural gene. The sequence of the probe diverges from that of the templates analyzed at 80 nt upstream of the major MCK transcription initiaition site. Thus, all transcripts initiating upstream of nt −80 will protect the same-sized probe fragment, allowing an estimate of the total contribution of such transcripts to the pool of CAT-encoding mRNA. Densitometric scanning of autoradiograms was used to obtain the percentage of transcripts in each category described in Results.

Sequencing was performed by the dideoxy chain termination method (42, 56) with either subclones in M13 vectors or single-stranded templates from pUC118-derived plasmids.

RESULTS

Deletion analysis of MCK 5′ flanking DNA. Previous work had shown that a fusion gene, containing 3,300 nt of MCK upstream sequence fused with the CAT structural gene at nt +7 (7 nt downstream of the MCK transcription start site), gave high-level expression specifically in differentiated MM14 muscle cultures (28). Unidirectional deletions of this fusion gene were made so that the same vector sequences were present, in each case, immediately upstream of the remaining MCK sequences. Proliferating myoblast cultures were cotransfected with each of these deletion plasmids and a reference plasmid and switched to differentiation medium. Cultures were harvested 24 h later and assayed for CAT and reference gene activity. The possibility of competition between the cotransfected plasmids for transcription factors was minimized by transfection with an amount of DNA which was well within the linear range for expression versus amount of DNA (data not shown).

Deletion to nt −1256 had little or no effect on expression in differentiated muscle cells (Fig. 1). Deletion to nt −1020, however, reduced expression to about 3% of the maximum level. Further deletion to nt −776 resulted in an additional small decrease in expression. Removal of all but 80 nt of 5′ flanking DNA gave an activity of 1%, which is about three times the background level of expression observed when all MCK sequences are removed. (The MCK gene contains the usual TATA sequence at nt −30, but does not appear to have a CAAT homology near nt −80 [28].) The residual activity without MCK sequences (p118CAT), although low, was still readily detectable and presumably resulted from transcription initiating within vector DNA. No CAT activity was observed in nontransfected cultures.

Statistical analyses (F-ratio tests) were performed on the data for constructs that expressed at low levels. These analyses showed that the mean values were significantly different between −776MCKCAT and −80MCKCAT (at the $P < 0.02$ confidence level) and between −80MCKCAT and p118CAT ($P < 0.01$), but not between −1020MCKCAT and −776MCKCAT ($P > 0.05$).

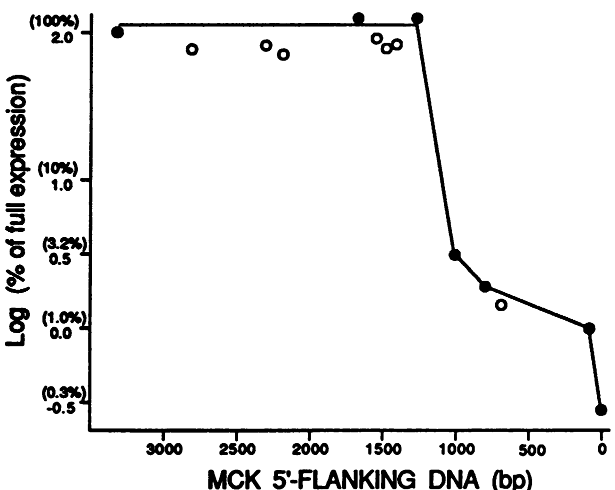

FIG. 1. Deletion analysis of MCK-CAT fusion genes. MCK DNA 5′ deletions were constructed in the plasmid −3300MCKCAT which has 3,300 nt of MCK 5′ flanking sequences fused near the transcription start site (nt +7) to the CAT structural gene. Proliferating MM14 cultures were transfected with MCK-CAT plasmid DNA and then switched to fibroblast growth factor-free differentiation medium and harvested 24 h later. CAT specific activity was corrected for variations in transfection efficiency by normalization to the specific activity of a cotransfected reference gene (β-galactosidase). Data are plotted on a semilog scale, with 100% defined as the expression level of −3300MCKCAT. Solid symbols represent constructs containing 3,300, 1,674, 1,256, 1,020, 776, 80, and 0 nt of MCK 5′ flanking DNA, and open symbols represent constructs containing 2,800, 2,300, 2,200, 1,510, 1,475, 1,409, and 723 nt of 5′ flanking DNA. Solid symbols represent mean values from at least seven transfections with at least two separate DNA preparations; open symbols represent mean values from at least two transfections. Significance levels for some of the smaller differences in expression are given in the text.

Position and orientation independence of the upstream element. Since the deletion analysis indicated that a sequence element between nt −1256 and −1020 was essential for high-level expression in differentiated muscle cells, subsequent studies have concentrated on characterizing this region. Two DNA fragments containing most of this upstream region were tested for the ability to enhance the expression of MCK promoters with either 80 or 776 nt of 5′ flanking sequence. Transient expression of several MCK-CAT constructs was first examined by using fully differentiated muscle cultures (Fig. 2, column 1). The larger upstream fragment (nt −1748 to −1050), when placed in its normal orientation immediately upstream of the nt −80 promoter (Fig. 2, plasmid 6), restored full expression. When placed in the opposite orientation, this fragment increased expression by a factor of 20 (compare plasmid 7 with plasmid 5), but restored only 20% of full expression. The smaller fragment (nt −1256 to −1050) also increased expression from the nt −80 promoter by a factor of 20 when placed downstream of the CAT gene, in either orientation (compare plasmids 8 and 9 with plasmid 5).

As mentioned previously, the nt −776 promoter (plasmid 10) exhibits more activity alone than does the nt −80 promoter (plasmid 5), and when either upstream fragment is placed downstream of the CAT gene (plasmids 11 to 14), the nt −776 promoter is 40 to 75% as active as the entire nt 3300 MCK 5′ region. On the basis of their relatively orientation- and position-independent activity, the upstream fragments (nt −1748 to −1050 and nt −1256 to −1050) appear to contain a transcriptional enhancer.

Multiple *cis*-acting sequences contribute to the specificity of MCK expression. To determine the extent to which the upstream enhancer contributes to the differentiation specificity of MCK expression (i.e., expression in differentiated muscle cultures versus that in proliferating cultures), these constructs were tested for their inducibility during MM14 differentiation. The results (Fig. 2, column 2) indicate that inducibility of the nt −80 promoter (plasmid 5) is low and is in fact in the range seen for non-muscle-specific promoters (see Fig. 4, plasmid 15 and 21). Induction was increased substantially by the presence of the MCK enhancer either upstream or downstream of the nt −80 promoter-structural gene unit (Fig. 2, plasmids 6 to 9). The nt −776 promoter (plasmid 10), without the enhancer, retained substantial inducibility, suggesting that DNA between nt −776 and −80 is contributing to the differentiation specificity of the endogenous gene (see Discussion). When the enhancer was present in addition to the nt −776 promoter, differentiation specificity was increased further (plasmids 11 to 14).

Initiation of transcription of MCK-CAT fusion genes at the normal MCK site. To determine the site(s) of transcription initiation in transfected MCK-CAT fusion genes, an S1 nuclease protection assay was performed. RNAs from differentiated cultures transcribing one of several highly active fusion genes were analyzed with a DNA probe end labeled within the CAT gene and extending 5′ of the endogenous MCK transcription start site (nt +1). At nt −80, the probe sequence diverges from that of the transfected constructs tested. Thus, specific initiation sites downstream of nt −80 will result in protection of discrete probe fragments, while all transcripts initiating upstream of nt −80 will protect a single-sized probe fragment (see Materials and Methods). The results show that the fusion genes give the same pattern of initiation, independent of the position and orientation of the enhancer (Fig. 3). A total of 80 to 90% of the transcripts initiate at the major site used by the endogenous MCK gene (28); 5 to 10% initiate 18 to 20 nt upstream of the major site, at a minor site also used in the endogenous gene (data not shown); and another 5 to 10% initiate upstream of nt −80. (There may also be a very small amount of initiation between 20 and 30 nt downstream of the major site.)

Enhancement of expression of heterologous promoters by the MCK upstream element in a cell-type-specific manner. To determine whether the MCK enhancer is capable of regulating expression from the promoters of non-muscle-specific genes, the two MCK enhancer-containing fragments described above were placed upstream of the herpes simplex virus thymidine kinase (TK) promoter, in either orientation. For comparison, the simian virus 40 enhancer (SV40), which is active in many cell types, was also placed in the same position. These constructs were tested for activity in three cell types: mouse L cells (nonmuscle), proliferating MM14 myoblasts, and differentiated MM14 muscle cells. The TK promoter had already been shown to be active in MM14 cells both before and after differentiation (41), and this result was confirmed with the TK-CAT construct in the present studies (Fig. 4, column 1, plasmid 15). The MCK enhancer had little or no effect on TK promoter activity in L cells (Fig. 4, plasmids 16 to 19; see below), whereas the SV40 enhancer-TK promoter construct (plasmid 20) was quite active in these cells. In differentiated muscle cells, however, the MCK enhancer increased expression from the TK promoter 10- to 40-fold (Fig. 4, column 2; compare plasmids 16 to 19 with plasmid 15), and the SV40 enhancer gave a similar result

	CONSTRUCT	1 RELATIVE EXPRESSION IN DIFFERENTIATED MUSCLE CELLS	2 FOLD INDUCTION DURING DIFFERENTIATION
1)	-3300 // -1748 E -1050 // CAT-poly(A)	100*	30 - fold*
2)	-1748 E -1050 // CAT-poly(A)	120 (12)	34 (7)
3)	-1256 E -1050 // CAT-poly(A)	120 (11)	32 (7)
4)	CAT-poly(A)	0.3 (8)	1.2 (7)
5)	-80 CAT-poly(A)	1.0 (9)	2.5 (7)
6)	E -80 CAT-poly(A)	130 (6)	29 (5)
7)	Ǝ CAT-poly(A)	20 (5)	33 (3)
8)	-80 CAT-poly(A) E	21 (4)	6.4 (4)
9)	-80 CAT-poly(A) Ǝ	25 (4)	10 (4)
10)	-776 CAT-poly(A)	1.9 (20)	13 (14)
11)	-776 CAT-poly(A) E	42 (8)	33 (6)
12)	-776 CAT-poly(A) Ǝ	74 (10)	25 (6)
13)	-776 CAT-poly(A) E	65 (6)	19 (4)
14)	-776 CAT-poly(A) Ǝ	63 (6)	21 (4)

FIG. 2. Effect of MCK upstream elements on expression in MM14 cells. DNA from the 5′ region of the MCK gene (nt −1748 to −1050 or nt −1256 to −1050) was cloned into various positions and orientations, as indicated (E represents the normal 5′-to-3′ orientation; upside-down E represents the opposite orientation). See Materials and Methods for details. Transfection and measurement of expression (given relative to that of −3300MCKCAT = 100) were as described in the legend to Fig. 1. Increase over proliferating levels is given as the average ratio of expression in parallel differentiated versus proliferating cultures. To correct for variations which occur in the background of differentiated cells that are present in proliferating cultures (this can be from 0.3 to 10%, depending on the quality of cell expansions prior to transfection), the ratios of expression in individual experiments were scaled to bring that of −3300MCKCAT (plasmid 1) to its mean induction level (30-fold, $n = 14$). The number of transfections is indicated in parentheses and always included at least two separate DNA preparations. Standard deviation for values reported for expression in differentiated muscle cells (column 1) for plasmids 2 to 14 were 30, 30, 0.1, 0.7, 20, 5, 3, 3, 0.9, 10, 25, 10, and 9, respectively; standard deviations for the values for fold induction (column 2) were 9, 8, 0.5, 1.7, 6, 8, 3.7, 2, 6, 17, 14, 16, and 4, respectively. Results of F-ratio statistical tests for differences between selected groups of construct pairs containing the larger and smaller MCK enhancers are reported in the text.

(plasmid 20). The MCK enhancer-TK promoter combination (plasmids 16 to 19; Fig. 4, column 3) exhibited differentiation specificity (high-level induction) similar to that of the entire MCK 5′ flanking region (plasmid 1), in contrast to the SV40 enhancer, which actually decreased the induction of expression from the TK promoter (compare plasmid 20 with plasmid 15).

The MCK enhancer also increased expression from the SV40 early promoter (SV_1) in differentiated MM14 cells, but showed no significant effect in L cells (Fig. 4, compare plasmid 21 with plasmids 22 to 25). However, this enhancement was less than with the TK promoter in differentiated muscle cells (compare plasmids 22 to 25 with plasmids 16 to 19). The MCK enhancer-SV40 promoter combination also exhibited less differentiation specificity (12- to 17-fold versus 22- to 36-fold induction; compare plasmids 22 to 25 with plasmids 16 to 19). This was true although the SV40 early promoter alone was induced more than the TK promoter alone (compare plasmid 21 with plasmid 15). Nevertheless, the MCK enhancer increased the differentiation specificity of both heterologous promoters, whereas the SV40 enhancer (plasmids 20 and 26) decreased it (by increasing expression more in proliferating cultures than in differentiated cultures).

Although the SV40 enhancer is much more active in L cells, the small MCK enhancer fragment did cause a significantly increased expression from the TK promoter in these cells ($0.01 < P < 0.05$; Fig. 4, compare plasmid 19 with plasmid 15), whereas the large MCK enhancer fragment did not (compare plasmids 16 and 17 with plasmid 15). This effect is consistent with small differences in the differentiation specificity (in MM14 cells) of the two MCK enhancer fragments in combination with either the TK or the MCK promoter and is considered more fully in the Discussion. In contrast, none of the MCK enhancer effects on the SV40 early promoter in L cells is statistically significant ($P > 0.05$).

DNA sequence surrounding the MCK enhancer. The upstream enhancer seems to be contained between nt −1256 and −1050 (Fig. 5). This sequence contains no core enhancer consensus, a sequence which has been found in several viral (26, 34, 73) and cellular (17) enhancers. It does, however, contain two sequences which are very similar to regions of the SV40 enhancer. One of these (Fig. 5, SV40-A) overlaps the SV40 core sequence and has been designated the *Sph*I region, and the other (SV40-B) corresponds to the TC-II region (75). Both of these SV40 regions have been shown to

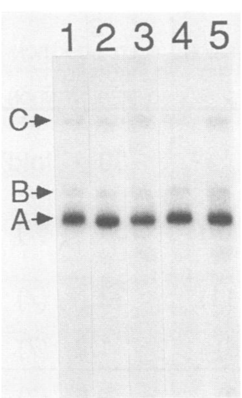

FIG. 3. Mapping of the transcription start site for transfected MCK-CAT plasmids. Myoblasts were transfected as described in the legend to Fig. 1 and then allowed to differentiate. Cells were harvested, and RNA was prepared 48 h after transfection. Transcripts were analyzed as described in Materials and Methods. This S1 protection analysis gives a band at position A for transcripts initiated at the primary MCK start site and a band at position B for a minor site used in the endogenous gene (see text). Transcripts initiated anywhere upstream of nt −80 contribute to a band at position C. Lanes: 1, RNA from cultures transfected with enl(+)−80MCKCAT (Fig. 2, plasmid 6); 2, −776MCKCATen2(−) (plasmid 14); 3, −776MCKCATen2(+) (plasmid 13); 4, −3300 MCKCAT (plasmid 1); 5, −1256MCKCAT (plasmid 3). Approximately 30 μg of RNA was used for each lane.

be essential for in vivo activity (75), and both bind cellular factors which protect them from DNase I cleavage (12, 74). Regions similar to sequences in two cellular enhancers, the immunoglobulin heavy chain (Fig. 5, IgH) (1, 11) and the κ light chain (Fig. 5, IgK) (52) enhancers, are also indicated. The immunoglobulin heavy-chain region overlaps two similarities reported between it and the polyomavirus enhancer (1), while the κ region is contained within a highly conserved intron region (14) and shares a similar motif to the region of immunoglobulin heavy-chain similarity (18). More detailed analysis will reveal whether these sequences are critical to the function to the MCK enhancer. Comparisons were also made, but no similarities as close as those described above were found with the polyomavirus enhancer (69), the mouse pancreatic elastase I enhancer (23, 49), the rat I insulin enhancer (48), the metallothionein enhancer (30) and metal regulatory elements (64), and the Moloney murine leukemia virus enhancer (63).

DISCUSSION

Transfection studies with the MM14 skeletal muscle culture system have revealed that several *cis*-acting DNA elements are involved in MCK gene regulation. Deletion analysis of upstream DNA has identified an element at about nt −1100 (relative to the transcription start site, nt +1) which is critical for high-level expression. Removal of this element results in a 30-fold decrease in expression of a linked CAT structural gene in differentiated muscle cultures (Fig. 1). This element possesses the enhancer properties of position- and orientation-independent function. For example, when positioned downstream of the CAT structural gene (and 600 nt farther than normal from the site of transcription initiation) in either orientation, it retains more than 60% of its normal activity (Fig. 2, column 1, plasmids 12 to 14). Consistent with results of observation of other enhancers (2), transcripts from these rearranged genes initiate at the normal site relative to MCK 5′ flanking DNA (Fig. 3).

MCK enhancer activity is quite specific to differentiated muscle cells, even when combined with promoter elements from nonmuscle genes. When the enhancer is placed upstream of the herpes simplex virus TK promoter, it increases expression up to 40-fold in differentiated muscle cells (Fig. 4, column 2, plasmids 16 to 19), yet it does not enhance expression in mouse L cells, a nonmyogenic cell line (column 1, plasmids 16 and 17). In contrast, the SV40 enhancer increases L-cell expression of the TK promoter 15-fold (plasmid 20). Since the MCK enhancer has very little effect on TK promoter activity in proliferating MM14 cells, this enhancer-promoter combination is dramatically induced during MM14 differentiation (column 3, compare plasmids 16 to 19 with plasmid 15). On the other hand, the SV40 enhancer is more active with the TK promoter in proliferating cells than in differentiated muscle cells (plasmid 20).

The MCK enhancer is also effective with the SV40 early promoter (SV_1), in that it increases expression in differentiated muscle cultures to a similar extent as does the homologous SV40 enhancer (Fig. 4, column 2, compare plasmids 22 to 25 with plasmid 26). As found with the TK promoter, the MCK and SV40 enhancers have opposite effects on the differentiation specificity of expression from SV_1; the MCK enhancer increases the ratio of expression in differentiated versus proliferating cultures (Fig. 4, column 3), whereas the SV40 enhancer decreases this ratio. There are distinct differences, however, between the two MCK enhancer-heterologous promoter combinations. The SV_1 promoter is enhanced to a lesser extent than the TK promoter, and the enhancement is less specific to differentiated muscle cultures. These differences are perhaps related to the fact that the SV_1 promoter is, by itself, induced significantly during muscle differentiation. These observations are consistent with the idea that the specificity of expression produced by a combination of regulatory elements is not determined by a simple addition of the specificities of the individual elements (55).

The specificity of the entire MCK 5′ flanking region appears to involve more than the enhancer element. A promoter with 776 nt of 5′ flanking DNA, without the enhancer, retains a strong inducibility during MM14 differentiation, even though its level of expression is drastically reduced (Fig. 2, plasmid 10). The nt −776 MCK promoter is expressed only twofold more strongly in differentiated muscle cells than is the nt −80 promoter (plasmid 5). However, its inducibility during differentiation is about five times greater (compare plasmid 10 with plasmid 5), because its level of expression in proliferating cultures is lower than that of the nt −80 promoter. Thus, the MCK region between nt −776 and −80 appears to have inhibitory effects in myoblast cultures (discussed further below). The differentiation specificity of both promoters is increased when the MCK enhancer is present (compare plasmids 11 to 14 with plasmid 10, and compare plasmids 6 to 9 with plasmid 5), consistent with its effect on heterologous promoters.

The difference between these two promoters, by themselves and with the MCK enhancer, suggests that the region between nt −776 and −80 also contains regulatory sequences. An alternative explanation is that the function of an element downstream of nt −80 is affected by the close proximity of vector sequences. In this interpretation, the additional proximal sequences (nt −776 to −80) serve only as a spacer. If true, this might also explain why the large enhancer fragment, when placed upstream (Fig. 2, plasmids

	CONSTRUCT	1 expression in mouse L cells	2 expression in differentiated muscle cells	3 fold induction during muscle differentiation
1)	-3300 // (-1748) E (-1050) // CAT-poly(A)	0.08 (3)	15 (15)	30 - fold*
4)	CAT-poly(A)	0.19 (2)	0.05 (7)	1.2 (7)
15)	TK CAT-poly(A)	1.0*	1.0*	2.7 (10)
16)	E TK CAT-poly(A)	1.2 (4)	24 (8)	26 (5)
17)	Ǝ TK CAT-poly(A)	1.0 (4)	12 (8)	36 (5)
18)	E TK CAT-poly(A)	2.5 (6)	28 (9)	22 (5)
19)	Ǝ TK CAT-poly(A)	3.5 (4)	42 (2)	26 (2)
20)	SV40 TK CAT-poly(A)	15.4 (4)	50 (3)	0.4 (2)
21)	SV CAT-poly(A)	1.0*	1.0*	8.1 (3)
22)	E SV CAT-poly(A)	1.3 (2)	6.0 (4)	13 (3)
23)	Ǝ SV CAT-poly(A)	1.8 (2)	7.7 (5)	17 (3)
24)	E SV CAT-poly(A)	0.9 (4)	6.9 (4)	13 (2)
25)	Ǝ SV CAT-poly(A)	1.0 (4)	4.8 (4)	12 (2)
26)	SV40 SV CAT-poly(A)	6.8 (2)	7.4 (5)	3.8 (5)

FIG. 4. Effect of the MCK upstream enhancer on cell-type- and differentiation-specific expression from heterologous promoters. The MCK enhancer regions shown in Fig. 2 were cloned upstream of heterologous promoters (herpes simplex virus TK or the SV40 early promoter, SV_1, without the SV40 enhancer) in both orientations. These were tested for expression as described in the legend to Fig. 1. Activities (for expression in mouse L cells and differentiated muscle cells) were scaled relative to that of the basal TK (upper part of figure) or SV40 early (lower part) promoter, set to 1.0 (asterisk). The basal level of expression from the SV40 early promoter (plasmid 21) in differentiated muscle cultures was 50% of that from the TK promoter (plasmid 15). Fold inductions were determined by comparing expression in differentiated MM14 cultures with that in proliferating cultures as described in the legend to Fig. 2. The SV40 enhancer, indicated as SV40 (which is active in most cell types), was used as a positive control. Standard deviations for values for plasmids 1, 4, 16 to 20, and 22 to 26 reported for expression in mouse L cells (column 1) were 0.08, 0.09, 0.6, 0.6, 1.3, 1.2, 7.0, 0.1, 0.5, 0.2, 0.1, and 0.2, respectively; standard deviations for the same plasmids for expression in differentiated muscle cells (column 2) were 7, 0.02, 6, 3, 3, 8, 23, 2.0, 2.9, 1.0, 1.5, and 2.5, respectively; and standard deviations for plasmids 4 and 15 to 26 for fold induction (column 3) were 0.6, 1.1, 4, 12, 2, 1, 0.2, 3.2, 5, 7, 3, 4, and 2.0, respectively. Results of F-ratio statistical tests for differences between selected groups of construct pairs containing the larger and smaller MCK enhancers are reported in the text.

6 and 7), increases the inducibility so dramatically (however, see below). In any event, elements between nt −776 and +7 can produce differentiation-specific expression without the MCK enhancer. Further study is necessary to characterize the activity of this region.

A third element present immediately upstream of the MCK enhancer is also implicated in both its cell type and differentiation specificity. The effect of this element (nt −1748 to −1256) is cryptic in its normal position in the gene, since its deletion has little or no effect in transient expression assays (Fig. 2, plasmids 2 and 3). A small effect is observed, however, when this element accompanies the MCK enhancer in other positions. Then it appears to increase the specificity of action of the enhancer during muscle differentiation both with the MCK promoter (Fig. 2; compare fold induction of plasmids 11 and 12 with that of plasmids 13 and 14) and with heterologous promoters (Fig. 4; compare plasmids 16 and 17 with plasmids 18 and 19, and compare plasmid 23 with plasmid 25). Although this effect is generally small (less than twofold), it is consistently observed and is strongest when the element is between the enhancer and promoter. Statistical comparison of mean induction values of constructs containing the larger and smaller MCK enhancer in identical positions indicated that the differences were significant ($P < 0.01$).

```
-1256  CCACTACGGGTCTAGGCTGCCCATGTAAGGAGGCAAGGCCTGGGGACACC
                                             GCCTGGGGAC
                                             231 SV40-B 240

-1206  CGAGATGCCTGGTTATAATTAACCCAGACATGTGGCTGCCCCCCCCC
        GAGATGCATGCTT          CCCAGGCAGGTGGC
        96   SV40-A  104      315    IgK    328

-1159  CCCCAACACCTGCTGCCTGAGCCTCACCCCCACCCCGGTGCCTGGGTCTT
       CCACATGACCTGCTTCCTG
       14      IgH       32

-1109  AGGCTCTGTACACCATGGAGGAGAAGCTCGCTCTAAAAATAACCCTGTCC

-1059  CTGGTGGATC -1050
```

FIG. 5. Sequence similarities between MCK and other enhancers. Similarities to other enhancers are indicated: SV40-A (12), 11/13-nt match; SV40-B, 10/10-nt match; IgK (52), 12/14-nt match; IgH (1), 15/19-nt match. The MCK sequence is numbered relative to the transcription start site. Numbers next to the other enhancer sequences indicate their locations as given in the cited references.

The element from nt −1748 to −1256 may also have a negative effect on enhancement of the TK promoter in L cells (Fig. 4, compare plasmids 16 and 17 with plasmids 18 and 19). These effects on specificity, like those described above for the region from nt −776 to −80, are most readily explained by an inhibition of expression in proliferating myoblast cultures (and possibly L cells). Since these elements seem to have similar negative effects, it is perhaps not surprising that the region from nt −1748 to −1256 can be deleted from its normal position in the gene without significant consequence as long as the inhibitory proximal sequences (nt − 776 to −80) are present. This apparent redundancy, however, does not necessarily imply an entirely redundant function in vivo, since both inhibitory elements may be required to produce a completely silent endogenous gene in myoblasts, nonmuscle tissues, or both.

Negative regulation in myoblasts has also been invoked to explain apparent differences in the behavior of two other muscle cell lines with respect to transfected versus endogenous gene expression (44). Studies suggested that *trans*-activators of some muscle genes are constitutively expressed in C2C12 mouse muscle cells (but not in rat L6 myoblasts), since transfected copies were active in the proliferating C2C12 cultures, while the endogenous muscle genes were silent until after differentiation (44, 60). However, in proliferating MM14 myoblasts, expression from transfected MCK promoters is very low, reflecting the quiescent state of the endogenous gene. Our data also indicate that the (3,300-nt) MCK 5′ flanking region confers regulated expression in C2C12 myoblasts (Johnson et al., submitted), unlike the other muscle gene promoters alluded to above.

An enhancer element that is separable from other promoter elements has recently been found in intron 1 of the quail troponin I gene (33a), but analysis of muscle actin genes has so far not localized any such enhancer element (45, 47). Whether this implies that different *trans*-acting regulators are activating separate subsets of muscle genes will only become clear as more detailed analyses are completed on a number of these genes.

Sequence comparisons between the MCK enhancer and other enhancers (Fig. 5) have revealed some striking similarities. The SV40 enhancer (12) and the immunoglobulin heavy-chain (1) and κ light-chain (52) enhancers each share a different sequence motif with the MCK enhancer. The SV40 regions have been identified as important for enhancer function (75), and each has also been shown to be involved in protein binding (12, 74). The immunoglobulin heavy-chain region binds a factor which seems to be specific to the B-cell lineage (15), but the MCK sequence is different at several nucleotides which are higly invariant in a consensus of immunoglobulin enhancer motifs which includes this region (11). One possibility is that a related but distinct protein binds to the MCK version of this motif. Preliminary results with subregions of the MCK enhancer indicate that most or all of the activity in differentiated muscle cells is retained by a 115-nt fragment (nt −1207 to −1093) which contains three of these four sequence similarities. Additional functional tests and factor-binding assays are necessary to fully assess the significance of these similarities. It will also be of interest to compare the MCK enhancer sequence with that of other muscle-specific enhancers when they become available.

ACKNOWLEDGMENTS

We thank Brad Olwin, Richard Palmiter, Tom Wilkie, and Ted Young for their many constructive discussions and critical comments on the manuscript. We also thank Pat Noel for her excellent technical assistance and Jon Herriott for the use of his computer facilities.

This research was supported by a grant from the Muscular Dystrophy Association and by Public Health Service grant AM 18860 from the National Institutes of Health. J.N.B. was supported by National Institutes of Health Training Grant GM07270, and J.E.J. was supported by National Institutes of Health Training Grant HD07183.

LITERATURE CITED

1. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
2. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
3. **Bergsma, D. J., J. M. Grichnik, L. M. A. Gossett, and R. J. Schwartz.** 1986. Delimitation and characterization of *cis*-acting DNA sequences required for the regulated expression and transcriptional control of the chicken skeletal α-actin gene. Mol. Cell. Biol. **6**:2462–2475.
4. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–613.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Buckingham, M. E.** 1977. Muscle protein synthesis and its control during the differentiation of skeletal muscle cells *in vitro*. Int. Rev. Biochem. **15**:269–332.
7. **Caravatti, M., A. Minty, B. Robert, D. Montarras, A. Weydert, A. Cohen, P. Daubas, and M. Buckingham.** 1982. The accumulation of messenger RNAs coding for muscle-specific proteins during myogenesis in a mouse cell line. J. Mol. Biol. **160**:59–76.
8. **Chamberlain, J. S., J. B. Jaynes, and S. D. Hauschka.** 1985. Regulation of creatine kinase induction in differentiating mouse myoblasts. Mol. Cell. Biol. **5**:484–492.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
10. **Chu, G., and P. A. Sharp.** 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. Gene **13**:197–202.
11. **Church, G. M., A. Ephrussi, W. Gilbert, and S. Tonegawa.** 1985. Cell-type-specific contacts to immunoglobulin enhancers in nuclei. Nature (London) **313**:798–801.

11a. **Clegg, C. H., and S. D. Hauschka.** 1987. Heterokaryon analysis of muscle differentiation: regulation of the postmitotic state. J. Cell Biol. **105**:937–947.

11b. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G_1 phase and is repressed by fibroblast growth factor. J. Cell Biol. **105**:949–956.

12. **Davidson, I., C. Fromental, P. Augereau, A. Wildeman, M. Zenke, and P. Chambon.** 1986. Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. Nature (London) **323**:544–548.
13. **Devlin, R. B., and C. P. Emerson, Jr.** 1979. Coordinate accumulation of contractile protein mRNAs during myoblast differentiation. Dev. Biol. **69**:202–216.
14. **Emorine, L., M. Kudhl, L. Weir, P. Leder, and E. E. Max.** 1983. A conserved sequence in the immunoglobulin J_K-C_K intron: possible enhancer element. Nature (London) **304**:447–449.
15. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. Science **227**:134–140.
16. **Gillies, S. D., V. Folsom, and S. Tonegawa.** 1984. Cell type-specific enhancer element associated with a mouse MHC gene, E_β. Nature (London) **310**:594–597.
17. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene.

Cell 33:717–728.

18. **Gimble, J. M., D. Levens, and E. E. Max.** 1987. B-cell nuclear proteins binding in vitro to the human immunoglobulin κ enhancer: localization by exonuclease protection. Mol. Cell. Biol. 7:1815–1822.
19. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA 79:6777–6781.
20. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
21. **Grichnik, J. M., B. J. Bergsma, and R. J. Schwartz.** 1986. Tissue restricted and stage-specific transcription is maintained within 411 nucleotides flanking the 5′ end of the chicken α-skeletal actin gene. Nucleic Acids Res. 14:1683–1701.
22. **Grosschedl, R., and D. Baltimore.** 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell 41:885–897.

22a. **Hammer, R. E., R. Krumlauf, S. A. Camper, R. L. Brinster, and S. M. Tilghman.** 1987. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. Science 235:53–58.

23. **Hammer, R. E., G. H. Swift, D. M. Ornitz, C. J. Quaife, R. D. Palmiter, R. L. Brinster, and R. J. MacDonald.** 1987. The rat elastase I regulatory element is an enhancer that directs correct cell specificity and developmental onset of expression in transgenic mice. Mol. Cell. Biol. 7:2956–2967.
24. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557–580.
25. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653–662.
26. **Herr, W., and J. Clarke.** 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. Cell 45:461–470.
27. **Imperiale, M. J., L. T. Feldman, and J. R. Nevins.** 1983. Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in *trans* and by a *cis*-acting adenovirus enhancer element. Cell 35:127–136.
28. **Jaynes, J. B., J. S. Chamberlain, J. N. Buskin, J. E. Johnson, and S. D. Hauschka.** 1986. Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol. Cell. Biol. 6:2855–2864.
29. **Kadesch, T., and P. Berg.** 1986. Effects of the position of the simian virus 40 enhancer on expression of multiple transcription units in a single plasmid. Mol. Cell. Biol. 6:2593–2601.
30. **Karin, M., A. Haslinger, A. Heguy, T. Dietlin, and T. Cooke.** 1987. Metal-responsive elements act as positive modulators of human metallothionein-II_A enhancer activity. Mol. Cell. Biol. 7:606–613.
31. **Karin, M., A. Haslinger, H. Holtgreve, G. Cathala, E. Slater, and J. D. Baxter.** 1984. Activation of a heterologous promoter in response to dexamethasone and cadmium by metallothionein gene 5′-flanking DNA. Cell 36:371–379.
32. **Klarsfeld, A., P. Daubas, B. Bourachot, and J. P. Changeux.** 1987. A 5′-flanking region of the chicken acetylcholine receptor α-subunit gene confers tissue specificity and developmental control of expression in transfected cells. Mol. Cell. Biol. 7:951–955.
33. **Konieczny, S. F., and C. P. Emerson, Jr.** 1985. Differentiation, not determination, regulates muscle gene activation: transfection of troponin I genes into multipotential and muscle lineages of 10T1/2 cells. Mol. Cell. Biol. 5:2423–2432.

33a. **Konieczny, S. F., and C. P. Emerson, Jr.** 1987. Complex regulation of the muscle-specific contractile protein (troponin I) gene. Mol. Cell. Biol. 7:3065–3075.

34. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79:6453–6457.
35. **Lassar, A. B., B. M. Paterson, and H. Weintraub.** 1986. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. Cell 47:649–656.
36. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) 325:368–372.
37. **Linkhart, T. A., C. H. Clegg, and S. D. Hauschka.** 1980. Control of mouse myoblast commitment to terminal differentiation by mitogens. J. Supramol. Struct. 14:483–498.
38. **Linkhart, T. A., C. H. Clegg, and S. D. Hauschka.** 1981. Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromolecular growth factors in the culture medium. Dev. Biol. 86:19–30.
39. **Medford, R. M., H. T. Nguyen, and B. Nadal-Ginard.** 1983. Transcriptional and cell cycle-mediated regulation of myosin heavy chain gene expression during muscle cell differentiation. J. Biol. Chem. 258:11063–11073.
40. **Melloul, D., B. Aloni, J. Calvo, D. Yaffe, and U. Nudel.** 1984. Developmentally regulated expression of chimeric genes containing muscle actin DNA sequences in transfected myogenic cells. EMBO J. 3:983–990.
41. **Merrill, G. F., S. D. Hauschka, and S. L. McKnight.** 1984. *tk* enzyme expression in differentiating muscle cells is regulated through an internal segment of the cellular *tk* gene. Mol. Cell. Biol. 4:1777–1784.
42. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19:269–276.
43. **Miksicek, R., A. Heber, W. Schmid, U. Danesch, G. Posseckert, M. Beato, and G. Schutz.** 1986. Glucocorticoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. Cell 46:283–290.
44. **Minty, A., H. Blau, and L. Kedes.** 1986. Two-level regulation of cardiac actin gene transcription: muscle-specific modulating factors can accumulate before gene activation. Mol. Cell. Biol. 6:2137–2148.
45. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. 6:2125–2136.
46. **Nadal-Ginard, B.** 1978. Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15:855–864.
47. **Nudel, U., D. Greenberg, C. P. Ordahl, O. Saxel, S. Neuman, and D. Yaffe.** 1985. Developmentally regulated expression of a chicken muscle-specific gene in stably transfected rat myogenic cells. Proc. Natl. Acad. Sci. USA 82:3106–3109.
48. **Ohlsson, H., and T. Edlund.** 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell 45:35–44.
49. **Orntiz, D. M., R. D. Palmiter, R. E. Hammer, R. L. Brinster, G. H. Swift, and R. J. MacDonald.** 1985. Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. Nature (London) 313:600–603.
50. **Osborne, T. F., D. N. Arvidson, E. S. Tyau, M. Dunsworth-Browne, and A. J. Berk.** 1984. Transcription control region within the protein-coding portion of adenovirus E1A genes. Mol. Cell. Biol. 4:1293–1305.
51. **Payvar, F., D. DeFranco, G. L. Firestone, B. Edgar, O. Wrange, S. Okret, J. Gustafsson, and K. R. Yamamoto.** 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35:381–392.
52. **Picard, D., and W. Schaffner.** 1984. A lymphocyte-specific enhancer in the mouse immunoglobulin κ gene. Nature (London) 307:80–82.
53. **Plon, S. E., and J. C. Wang.** 1986. Transcription of the human β-globin gene is stimulated by an SV40 enhancer to which it is physically linked but topologically uncoupled. Cell 45:575–580.
54. **Ptashne, M.** 1986. Gene regulation by proteins acting nearby and at a distance. Nature (London) 322:697–701.
55. **Robbins, P. D., D. C. Rio, and M. R. Botchan.** 1986. *trans*-Activation of the simian virus 40 enhancer. Mol. Cell. Biol.

6:1283–1295.

56. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
57. **Sassone-Corsi, P., and I. M. Verma.** 1987. Modulation of c-*fos* gene transcription by negative and positive cellular factors. Nature (London) **326:**507–510.
58. **Schafer, D. A., J. B. Miller, and F. E. Stockdale.** 1987. Cell diversification within the myogenic lineage: *in vitro* generation of two types of myoblasts from a single myogenic progenitor cell. Cell **48:**659–670.
59. **Seed, J., and S. D. Hauschka.** 1984. Temporal separation of the migration of distinct myogenic precursor populations into the developing chick wing. Dev. Biol. **106:**389–393.
60. **Seiler-Tuyns, A., J. D. Eldridge, and B. M. Paterson.** 1984. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. Proc. Natl. Acad. Sci. USA **81:**2980–2984.
61. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eucaryotic gene transcription. Trends Genet. **1:**224–230.
62. **Shani, M., D. Zevin-Sonkin, O. Saxel, Y. Carmon, D. Katcoff, U. Nudel, and D. Yaffe.** 1981. The correlation between the synthesis of skeletal muscle actin, myosin heavy chain, and myosin light chain and the accumulation of corresponding mRNA sequences during myogenesis. Dev. Biol. **86:**483–492.
63. **Speck, N. A., and D. Baltimore.** 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. Mol. Cell. Biol. **7:**1101–1110.
64. **Stuart, G. W., P. F. Searle, H. Y. Chen, R. L. Brinster, and R. D. Palmiter.** 1984. A 12-base-pair motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA **81:**7318–7322.
65. **Swift, G. H., R. E. Hammer, R. J. MacDonald, and R. L. Brinster.** 1984. Tissue-specific expression of the rat pancreatic elastase I gene in transgenic mice. Cell **38:**639–646.
66. **Takahashi, K., M. Vigneron, H. Matthes, A. Wildeman, M. Zenke, and P. Chambon.** 1986. Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature (London) **319:**121–126.
67. **Taylor, S. M., and P. A. Jones.** 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell **17:**771–779.
68. **Treisman, R.** 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. Cell **42:**889–902.
69. **Tyndall, C., G. L. Mantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucleic Acids Res. **9:**6231–6250.
70. **Wang, X., and K. Calame.** 1986. SV40 enhancer-binding factors are required at the establishment but not the maintenance step of enhancer-dependent transcriptional activation. Cell **47:**241–247.
71. **Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon.** 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell **32:**503–514.
72. **Wasylyk, C., and B. Wasylyk.** 1986. The immunoglobulin heavy-chain B-lymphocyte enhancer efficiently stimulates transcription in non-lymphoid cells. EMBO J. **5:**553–560.
73. **Weiher, H., M. Konig, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219:**626–631.
74. **Wildeman, A. G., M. Zenke, C. Schatz, M. Wintzerith, T. Grundstrom, H. Matthes, K. Takahashi, and P. Chambon.** 1986. Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. **6:**2098–2105.
75. **Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5:**387–397.