MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4826–4836
0270-7306/90/094826-11$02.00/0

Vol. 10, No. 9

Brain and Muscle Creatine Kinase Genes Contain Common TA-Rich Recognition Protein-Binding Regulatory Elements

ROBERT A. HORLICK, GRACE M. HOBSON, JAMES H. PATTERSON, MARK T. MITCHELL, AND PAMELA A. BENFIELD*

E. I. du Pont de Nemours and Co., Inc., P.O. Box 80328, Wilmington Delaware 19880-0328

Received 22 February 1990/Accepted 7 June 1990

We have previously reported that the rat brain creatine kinase (*ckb*) gene promoter contains an AT-rich sequence that is a binding site for a protein called TARP (TA-rich recognition protein). This AT-rich segment is a positively acting regulatory element for the *ckb* promoter. A similar AT-rich DNA segment is found at the 3′ end of the 5′ muscle-specific enhancer of the rat muscle creatine kinase (*ckm*) gene and has been shown to be necessary for full muscle-specific enhancer activity. In this report, we show that TARP binds not only to the *ckb* promoter but also to the AT-rich segment at the 3′ end of the muscle-specific *ckm* enhancer. A second, weaker TARP-binding site was identified in the *ckm* enhancer and lies at the 5′ end of the minimal enhancer segment. TARP was found in both muscle cells (C2 and L6 myotubes) and nonmuscle (HeLa) cells and appeared to be indistinguishable from both sources, as judged by gel retardation and footprinting assays. The TARP-binding sites in the *ckm* enhancer and the *ckb* promoter were found to be functionally interchangeable. We propose that TARP is active in both muscle and nonmuscle cells and that it is one of many potential activators that may interact with muscle-specific regulators to determine the myogenic phenotype.

We have been using the cytoplasmic creatine kinase (*ck*) genes as a model system to study differential gene regulation (4). The muscle *ck* gene (*ckm*) is expressed most prominently in differentiated skeletal and cardiac muscle tissue (51), although there are reports of significant expression in some nonmuscle tissues (21, 27). The brain *ck* gene (*ckb*) has wider tissue distribution and is expressed at differing levels in many tissues with the probable exception of liver (48). During skeletal muscle differentiation, *ckb* is expressed in myoblasts and is replaced by *ckm* when myoblasts fuse to form myotubes (10, 25, 38).

Up regulation of the *ckm* gene during myogenesis is controlled by at least two enhancer elements (47), one 5′ to the gene (24, 26) and one located in the first intron (47). The 5′ enhancer is the most completely characterized and appears to be composed of multiple regulatory elements (24).

Recently, a number of related muscle-specific factors that have the capacity to convert native 10T1/2 and a variety of nonmuscle cell types to the myogenic phenotype have been identified and cloned (7, 14, 17, 30, 39, 41, 52, 54). These factors include myd (39), myoD (14), myogenin (17, 54) myf 5 (7), and MRF4 (41). At least myoD, myogenin, MRF4, and myf 5 are structurally related (41) and contain a conserved helix-loop-helix motif believed to be important for heterodimer formation and DNA binding (36, 37). Together with myd, they are candidates for transcription factors important for tissue-specific expression of muscle-specific genes. Two binding sites for myoD have been located in the *ckm* enhancer (28). The 3′ most of these sites has also been described as binding a factor called mef1 (9). The exact relationship of mef1 to myoD and myogenin is unclear, although they appear to be antigenically related (9). Thus, it seems likely that myoD and myogenin may play a key role in *ckm* enhancer activity.

However, other elements in the *ckm* enhancer appear important for complete enhancer function, and myoD-binding sites cannot function alone to determine muscle specificity. An AT-rich region at the 3′ end of the enhancer is required in certain constructs for full enhancer activity (19, 24). This region, which we have previously termed E3, binds a nuclear factor (24), although there is disagreement in the literature as to whether this binding is muscle specific (9, 19, 24). Thus, the E3 region represents a potential candidate for binding of factors that may interact with myoD-like molecules to determine muscle specificity.

The *ckb* gene is turned off during skeletal muscle myogenesis, although it is coexpressed with the *ckm* gene in adult cardiac tissue (10, 38, 48). The *ckb* promoter initiates transcription by using a nonconsensus TATA box (23; G. Hobson, G. Molloy, and P. A. Benfield, submitted for publication). Upstream of this nonconsensus TATA box is a perfect consensus TATA sequence that appears not to function as a typical TATA box in vivo but rather serves as a *cis*-acting positive regulator of the downstream nonconsensus TATA box (23; Hobson et al., submitted). This upstream TATA sequence binds a factor called TA-rich recognition protein (TARP) that is distinct from TFIID. TARP is a candidate for a positively acting transcription factor for the *ckb* gene (23). The TARP-binding sequence shows sequence similarity to the AT-rich sequence shown to be important for *ckm* enhancer function.

In this report, we examine the ability of TARP to interact with sequences in both the *ckm* enhancer and the *ckb* promoter. We also examine the possibility that these sequences represent common regulatory elements shared between the two genes and propose that TARP may be able to interact with other regulators, e.g., myoD, and potentially with itself to control muscle-specific transcription.

* Corresponding author.

CCTGGTTATAATTAACCTGGACACGTGGTTGCTCCC	RAT E1
TTAACCTGGACACGTGGTTGCTCCCCCCC	RAT MEDIUM E1
CTGGACACGTGGTTGCTCCCC	RAT SHORT E1
TGCCTGGTTATAATTAACCTGGA	RAT E3'
AAGCTTGCTCTAAAAATAACTCTATCCC	RAT E3
TCAGCCCCTGGGGCAGCCCATACAAGG	RAT BOX a
CCTTGGCTCCATGAATGGCCTCGG	CArG1
GCGAAGGGGACCAATTAAGGCAAGGTGGC	CArG2
CATCTTTTAAAAATAACTTTTCAAA	MLC 1/3
AGGCTAAAAATAACCCCATGTCCAC	MLC2
CCCTCCCTGGGGACAGCCCCTCCTGGC	NON. SP.
GAATGAATGGGCTATAAATAGCCGCCAATGGGAGGCCGGCGACGC	CKBTATA

FIG. 1. Sequences of oligonucleotides used as probes or competitors in gel retardation assays.

MATERIALS AND METHODS

Preparation of extracts. Nuclear extracts from C2 myotubes and from HeLa cells were prepared according to the procedure of Shapiro et al. (43). Nuclear extracts from L6 myoblasts and myotubes were prepared according to the procedure of Dignam et al. (15). Extracts from brain tissue were prepared as previously described (23).

Gel retardation assays. Gel retardation assays were performed by a modification of the procedure of Singh et al. (44) as previously described (23, 24). The *ckm* enhancer probe was prepared as follows. Plasmid Xba 1.5 (24), which contains 1.4 kilobase pairs upstream of the rat *ckm* transcript start point linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, was digested with *Bam*HI and *Stu*I. In this way, a 159-base-pair (bp) fragment was generated that ran from the *Bam*HI site at −1301 to the *Stu*I site at −1190 and included the entire minimal enhancer fragment. Fragments and oligonucleotide probes were labeled by treatment with polynucleotide kinase in the presence of [γ-^{32}P]ATP. Alternatively, oligonucleotides were labeled by filling in two complementary overlapping oligonucleotides, using the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dTTP.

Oligonucleotides used as competitors in gel retardation assays are shown in Fig. 1.

In vitro transcription reactions. In vitro transcription reactions were performed by using HeLa nuclear extracts as described previously (23). RNA products were analyzed by primer extension as described below.

Preparation of constructs. Wild-type and mutant versions of the *ckb* promoter were inserted upstream of the neomycin resistance (*neo*) gene in the plasmid vector pNVD. Plasmid pNVD is derived from pUC^{PL}CAT (5) by replacement of the CAT gene with the *neo* gene. In addition, the adenovirus VA gene is inserted into the vector downstream of the *neo* gene and in the opposite orientation. Transcription from the adenovirus VA gene by RNA polymerase III provides an internal control for transient gene transfer and in vitro transcription experiments. Wild-type and mutant *ckb* promoter segments were generated synthetically by ligation of a series of synthetic oligonucleotides. These constructs will be described in detail elsewhere (Hobson et al., submitted). Briefly, promoter segments were designed to insert upstream of the *neo* gene between a 5′ *Hin*dIII site and a 3′ *Bgl*II site. The construct was designed to include *ckb* promoter sequence from −195 to +4 in such a way as to reconstruct the correct transcript start points for the *ckb* gene.

Constructs that link the wild-type and mutant *ckm* promoter-enhancer segments upstream of the bacterial CAT gene were prepared in pUC^{PL}CAT (5). A synthetic modular enhancer was generated to facilitate mutation of individual enhancer elements. Plasmid Xba 1.5 (24) was digested with *Aat*II and *Bsp*MI. This digestion cuts at the *Bsp*MI site at position −1120 in the *ckm* enhancer and also upstream of the enhancer at the *Aat*II site at position 2617 in the pUC vector. The enhancer was reconstructed in a triple ligation using two pairs of oligonucleotides as follows.

*Aat*II 5′-CCGAGATGCCTGGTTATAATTAACCTGGACACGTGGTTGC *Xho*I
TGCAGGCTCTACGGACCAATATTAATTGGACCTGTGCACCAACGAGCT-5′

*Xho*I 5′-TCGAGCCCCCCAACACCTGCTGCC *Bsp*MI
CGGGGGGTTGTGGACGACGGACTG-5′

This places a synthetic *Xho*I site at position −1139 in the enhancer and introduces four extra bases (shown in bold type). These sites are introduced at a location where the rat and mouse *ckm* enhancer sequences differ by four bases. In this way, the spacing within the rat *ckm* enhancer is changed to that reported for the mouse *ckm* enhancer (26). This mutation has no effect on enhancer function (P. Benfield, unpublished observations). A new enhancer is generated that runs from −1031 to −1179 (−1184 in new spacing). The *Aat*II site at −1179 and the *Xho*I site at −1139 are synthetic and not found in the native enhancer. Introduction of these restriction sites facilitates mutagenic manipulation of the 5′ end of the enhancer segment. The 5′ endpoint of this enhancer segment is such that the simian virus 40B and CArG homologies (24, 26, 47) are no longer present. Mutations in the E3 region of this modular enhancer were created by digestion with *Hin*dIII and *Bam*HI to remove the native E3 region. Synthetic oligonucleotides were then inserted between the *Hin*dIII (−1063) and *Bam*HI (−1031) sites to create desired mutations (see Fig. 7). The region between the *Bam*HI site at −1031 and the *Nhe*I site at −480 was removed by digestion with *Bam*HI and *Nhe*I, blunt ending with T4 DNA polymerase, and ligation with T4 DNA ligase. Similarly, deletion of the entire E3 region was achieved by digestion with *Hin*dIII, which cleaves at position −1063 upstream of the E3 region, and *Nhe*I, blunt ending with T4 DNA polymerase, and ligation with T4 DNA ligase.

Gene transfer. HeLa cells (a kind gift of Richard Morimoto) were grown in monolayer culture in Dulbecco modified Eagle medium with 10% fetal calf serum and 50 μg of gentamicin per ml. Calcium phosphate-mediated gene transfer was performed as previously described (20, 53). Cells were plated at a density of 10^5 cells per 100-mm dish and allowed to grow for 48 h before transfection. Calcium phosphate-precipitated DNA (15 μg per dish) was pipetted on the cells (two dishes per construct) and allowed to remain for 18 h. The DNA was removed from the cells by feeding with fresh medium; 24 h later, RNA was harvested for analysis.

Gene transfer into C2 myogenic cells (55), harvesting of cell extracts, and CAT assays were performed as previously described (24). Each construct was assayed at least three times. Transfer experiments were performed by using 20 μg of test plasmid DNA plus 5 μg of plasmid pCH110 (Pharmacia, Inc., Piscataway, N.J.) as an internal control. Plasmid

pCH110 contains the simian virus 40 promoter-enhancer combination driving the β-galactosidase gene. β-galactosidase activity was measured as described previously (24). [^{14}C]chloramphenicol and its acetylated forms were separated by thin-layer chromatography (18) and quantitated by using the AmBis radioanalytic imaging system (AmBis Systems, San Diego, Calif.).

RNA isolation and analysis. RNA was prepared according to Chirgwin et al. (11) and quantitated by measuring the A_{260}.

Primer extension analysis was carried out essentially as described by Hobson et al. (23). A 0.1-ng (100,000-cpm) sample of 5′-^{32}P-end-labeled oligonucleotide 5′-CGTGCAA TCCATCTTG-3′ (bases 26 to 41 from the translation start site of the *neo* gene) or oligonucleotide 5′-CGCCATGATA CCCTTGCG-3′ (bases 32 to 49 from the transcription start site of the VA gene) was mixed with 10 or 20 μg of RNA in a total volume of 6 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM disodium EDTA. The mixture was heated above 70°C in a water bath and slowly cooled to room temperature. The reaction was then adjusted to 0.5 mM each dATP, dCTP, dGTP, and dTTP–10 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$–50 mM KCl–1 mM dithiothreitol–11 U of reverse transcriptase (Dupont, NEN Research Products, Boston, Mass.) in a total volume of 10 μl and incubated at 42°C for 1 h. The reaction mix was adjusted to 150 mM NaCl and ethanol precipitated. The precipitate was suspended in formamide load dye. Half of each sample was electrophoresed on an 8 M urea–7% polyacrylamide sequencing gel. Products were quantitated by using an AmBis radioanalytic imaging system.

Footprinting. DNase I footprinting experiments were performed on the *ckb* promoter by a modification of the procedure of Babiss et al. (2) as previously described (23). A *Hin*fI-*Pst*I fragment was used as a probe. This fragment was 5′ end labeled on the noncoding strand, using polynucleotide kinase and [γ-^{32}P]ATP. Reactions contained 12 to 30 μg of L6 myoblast or myotube extract and 5 μg of sonicated *Escherichia coli* DNA as competitor. The final DNase I concentration was 10 μg/ml.

RESULTS

We have previously defined a minimal 5′ enhancer segment for the rat *ckm* gene. This 159-bp enhancer segment includes at least three nuclear protein-binding domains (E1, E2, and E3) that can be identified by DNase I footprinting. Binding to the E1 core region can be detected in gel retardation assays using nuclear extracts from many sources. With use of HeLa nuclear extracts and the entire minimal enhancer segment as a probe, a retarded species was detected that could be abolished with a synthetic oligonucleotide that corresponds to the E1 core sequence. Higher-resolution gel retardation assays revealed that this retarded species was composed of a closely spaced triplet of bands (Fig. 2). To investigate the origin of these bands, competition experiments were performed with several shorter oligonucleotides derived from the E1 core region. All three bands were removed in the presence of an oligonucleotide derived from the entire E1 core region (Fig. 2, lanes 2 to 4). Shorter oligonucleotides derived from the 3′ end of the E1 core (rat medium E1 and rat short E1) competed for only the two faster-migrating components (Fig. 2, lanes 5 to 9). Conversely, an oligonucleotide derived from the 5′ end of the E1 core (rat E3′) competed for only the most slowly migrating band (Fig. 2, lanes 13 to 15). These results suggested that the E1 core region represented binding sites for at least two proteins. Consistent with this conclusion, incubation with a mixture of oligonucleotides E3′ and E1 short (Fig. 2, lane 16) resulted in removal of all three bands. The rat E3′ sequence contains within it an AT-rich stretch similar to that found in the E3 binding region at the 3′ end of the minimal enhancer. To determine whether these two sequences were related in their ability to bind nuclear proteins, the rat E3 oligonucleotide was used as a competitor in the gel retardation assay shown in Fig. 2. This oligonucleotide competed for the slowest-migrating retarded band (Fig. 2, lanes 10 to 12) in a manner similar to that described for rat E3′ (Fig. 2, lanes 13 to 15). For both E3 and E3′, competition for the slowest-migrating band resulted in increased intensity of the two faster-migrating bands. Competition with increasing amounts of each oligonucleotide suggest that the rat E3 sequence is about 10-fold stronger in its binding capacity for this factor than is rat E3′.

We have previously described a protein, TARP, that recognizes an AT-rich sequence in the rat *ckb* promoter (23). The TARP-binding site appears to be a positively acting *cis* regulatory sequence for *ckb* expression (Hobson et al., submitted) and is similar to the E3 and E3′ sequences in the *ckm* 5′ enhancer. To investigate the relationship between TARP and the two E3 regions, gel retardation experiments were performed, using either the *ckm* E3 region oligonucleotide or the *ckb* TARP-binding oligonucleotide (CKBTATA) as a probe. Retarded species B_2 and B_3 were generated on each probe with both HeLa and C2 myotube extracts (Fig. 3). The shifted bands generated with each extract were similar on each probe. However, band B_3 generated with C2 myotube extracts showed significant smearing. This smearing is very common in myotube extracts and varies between extracts. It may result from modification events, such as TARP proteolysis, occurring in myotube extracts or from the presence of multiple binding activities.

We compared the ability of a variety of synthetic oligonucleotides to compete for binding of each extract to each of the two probes. No competition was observed with box a, $CArG_1$, or $CArG_2$. Box a represents a sequence reiterated within the *ckm* promoter (25). The two CArG sequences represent two functional CArG boxes in the human cardiac actin promoter (32, 34). Several oligonucleotides, however, did compete effectively for B_3 binding with each extract on each probe. These included the rat *ckm* E3 sequence, the *ckb* promoter TARP recognition sequence, and an AT-rich sequence from the chicken myosin light-chain 2 promoter (LC2). The LC2 sequence corresponds to the distal promoter element shown to be necessary for muscle-specific regulation of the cardiac myosin LC2 promoter (1, 8). Weaker competition was observed with the E1 core sequence that contains within it the E3 sequence and with an AT-rich segment found within the rat myosin light-chain 1/3 3′ enhancer (LC1/3) (16).

To test further the relationship between the activity that recognizes the enhancer E3 region and the *ckb* TATA sequence, titrations with each of the competing oligonucleotides were performed (Fig. 4). Either the E3 or the CKBTATA oligonucleotide was used as a probe. Nuclear extracts were derived from HeLa cells. Three retarded bands, B_1, B_2, and B_3, were generated. B_1 and B_2 appeared to be nonspecific (Fig. 3 and 4) and probably resulted from single-stranded DNA-binding proteins that recognize contaminating single-stranded DNA in the oligonucleotide probes but not in fragment probes. However, band B_3 could be competed for by the oligonucleotides tested in a manner indicative of specific binding. All of the oligonucleotides

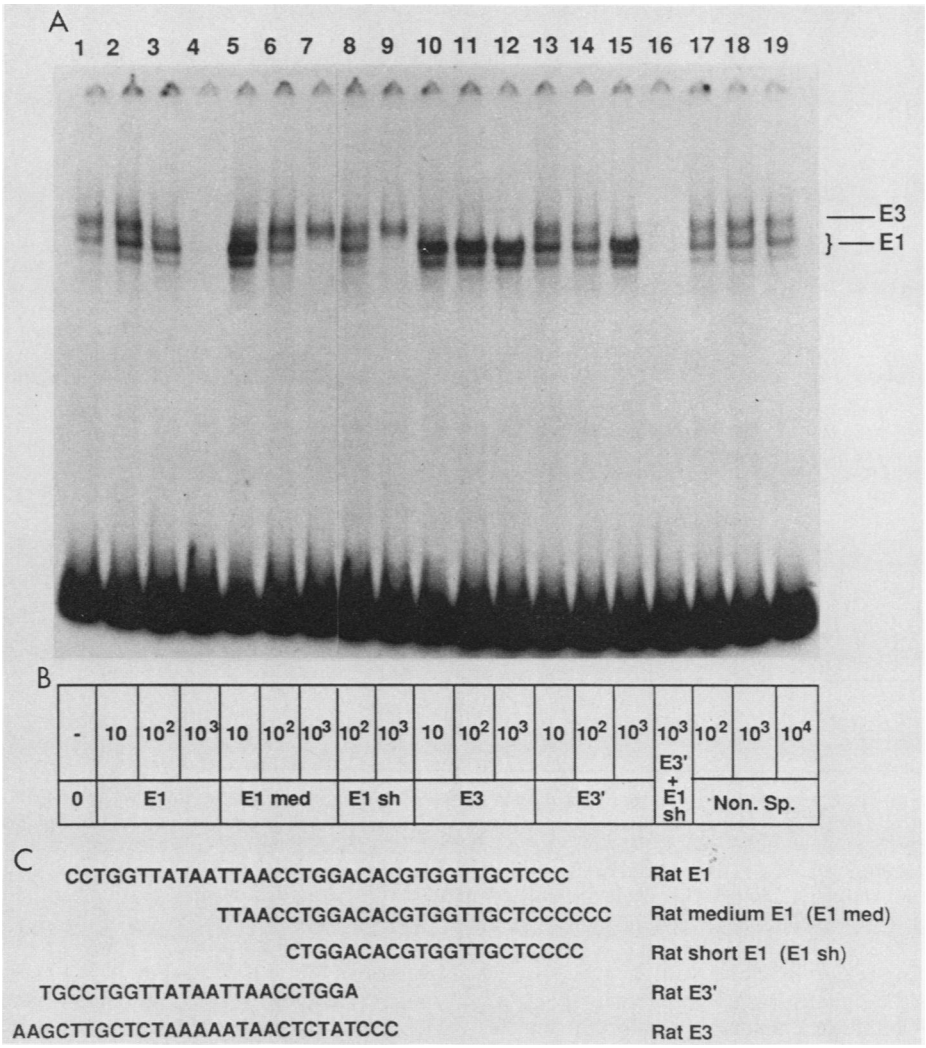

FIG. 2. (A) Gel retardation assay performed by using a 159-bp fragment derived from the rat *ckm* 5′ enhancer as a probe. The probe represents a fragment derived by restriction of the 5′ upstream region with *Bam*HI and *Stu*I and runs from −1031 to −1190. Positions of retarded bands that represent binding to the E1 and E3 regions of the enhancer are indicated on the right. The gel retardation assay was performed in the presence of cold oligonucleotides (B). (C) Sequences of the cold oligonucleotides. The sequence of the nonspecific oligonucleotide is shown in Fig. 1. The molar excess of oligonucleotide is indicated in the top portion of panel B. med, Medium; sh, short; Non. Sp., nonspecific.

tested (LC1/3, LC2, CKBTATA, and E3) showed the same titration profiles on both probes. The LC2, CKBTATA, and E3 oligonucleotides showed equivalent competition profiles. However, like the E3′ sequence, the LC1/3 oligonucleotide was approximately 10-fold weaker in its ability for compete for this binding activity (Fig. 4; compare lanes 2 and 3 with lanes 5 and 6). A list of known TARP-binding sites is shown in Fig. 5 together with a preliminary consensus sequence for TARP binding.

We next tested whether the E3 region of the *ckm* enhancer and the *ckb* TATA TARP-binding site were functionally interchangeable. We have previously shown that the *ckb* gene is expressed in HeLa cells; 200 bp of *ckb* promoter support expression in transient gene transfer assays in HeLa cells and in in vitro transcription assays using HeLa cell nuclear extracts. We have linked 200 bp of *ckb* promoter to a *neo* reporter gene in the plasmid vector pNVD. Constructs were prepared that replaced the CKBTATA TARP-binding site with the E3 region from the *ckm* enhancer so that the spacing of elements within the *ckb* promoter was maintained. Similarly, a linker scan mutation was created in the *ckb* promoter so that the TARP-binding sequence was replaced by a *Cla* linker (Fig. 6e). These constructs were introduced into HeLa cells to test their ability to support expression of the *neo* gene. RNA was harvested, and neomycin-resistant RNA and VA control RNA were analyzed by primer extension (Fig. 6a). The level of transcription of the *neo* gene relative to that of the VA gene is shown in Fig. 6b.

The linker scan mutation that destroys the *ckb* TARP-binding site (P. Harlow and P. A. Benfield, unpublished data) resulted in a two- to fivefold drop in relative transcription of the *neo* gene. Replacement of the *ckb* TARP-binding site by the enhancer E3 sequence restored *ckb* promoter activity. A similar result was obtained when these constructs were used as substrates for in vitro transcription reactions using HeLa cell nuclear extracts (Fig. 6c and d).

A similar replacement experiment was performed on the *ckm* enhancer. The E3 enhancer segment was replaced with either of two different linkers, Mut E3a and Mut E3b, or with

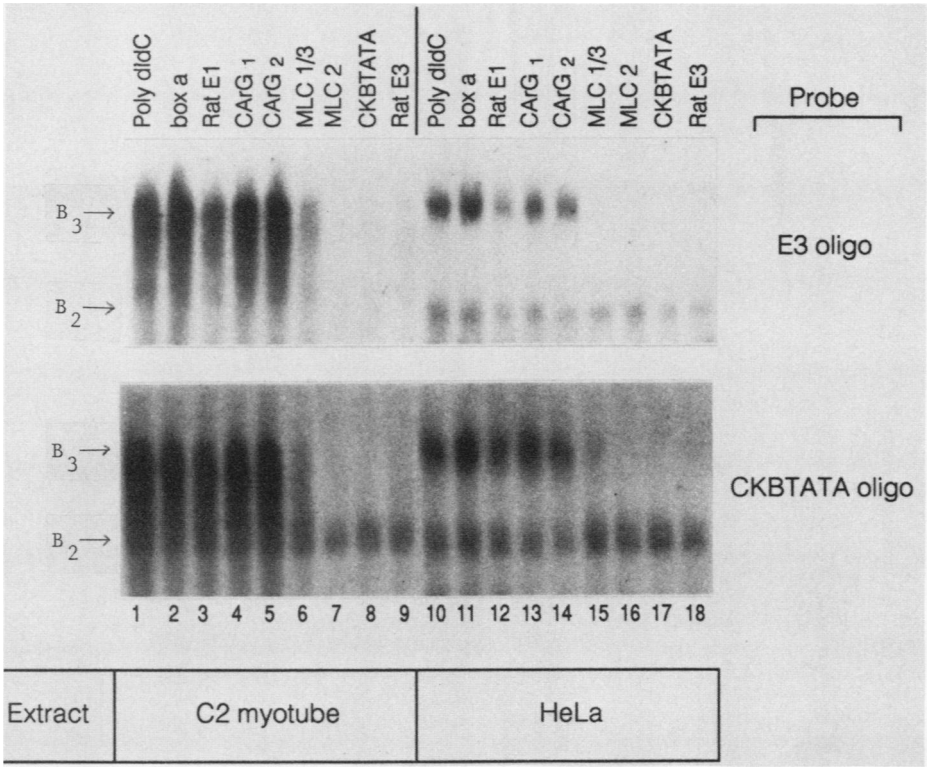

FIG. 3. Gel retardation assay performed by using [^{32}P]kinase end-labeled synthetic oligonucleotides E3 and CKBTATA as indicated. The experiment was performed by using nuclear extracts from C2 myotubes or from HeLa cells as shown at the bottom. Incubations were carried out in the presence of 3 μg of nonspecific poly(dI-dC) alone or together with a 1,000× excess of cold oligonucleotide as indicated above each lane. Only the portions of the gel containing retarded bands are shown. Band B_2 appears to be derived from nonspecific binding to the probe. Band B_3 is derived from specific binding to the oligonucleotide probes. MLC, Myosin light chain.

the *ckb* TARP-binding region in such a way that the spacing of elements within the enhancer was maintained (Fig. 7a). The ability of each of these enhancers to support muscle-specific up regulation of the *ckm* promoter linked to a reported CAT gene was tested by introduction into differentiating C2 myogenic cells (Fig. 7b).

The wild-type enhancer supported muscle-specific up regulation of the *ckm* promoter. Mutation or deletion of the E3 region resulted in significant loss of enhancer activity. Conversely, replacement of the E3 region with the *ckb* TARP-binding region resulted in restoration of enhancer activity. This finding suggests that the *ckb* TARP-binding region can functionally replace the E3 region in the *ckm* enhancer.

We have previously shown by footprinting analysis that TARP activity appears to be present in nuclear extracts from both brain tissue and HeLa cells (23). The results presented here (Fig. 3) suggest that TARP is also present in muscle cells. As a further confirmation of this possibility, we performed DNase I footprinting experiments on the *ckb* promoter, using nuclear extracts from brain and from L6 myoblasts and L6 myotubes (Fig. 8). Identical areas of protection covering the *ckb* upstream TATA box were observed with all three extracts, again suggesting that TARP-like activity is present in multiple cell types, including muscle.

DISCUSSION

We have previously described a factor, TARP, that recognizes an AT-rich segment in the *ckb* promoter region. Although this AT-rich segment is a perfect consensus TATA box, the protein TARP that binds to it appears to be distinct from the classic TATA box-binding factor TFIID (23, 40, 42). TARP shows a different temperature inactivation profile from TFIID and chromatographs differently on ion-exchange columns (M. T. Mitchell and P. A. Benfield, unpublished observations). The TATA box function for the *ckb* promoter is provided by a nonconsensus TATA box that lies about 20 bp downstream of the perfect consensus TATA box (Hobson et al., submitted). The upstream TATA sequence that binds TARP can serve as a TATA box and assemble a transcription initiation complex in vitro (23, 33). However, we have been unable to show that it is normally used as a TATA box in vivo (23). Rather, this sequence appears to serve as a positive *cis*-acting regulatory element for the *ckb* promoter at the downstream nonconsensus TATA box, and therefore TARP is a candidate for a positively acting transcription factor.

A similar AT-rich region (E3) at the 3′ end of the rat *ckm* 5′ minimal enhancer appears necessary for full enhancer function and is a binding site for a nuclear factor (24). We have shown in this report that the TARP protein that recognizes the *ckb* promoter also recognizes this E3 region. Furthermore, a second, 10-fold-weaker TARP-binding site is found at the 5′ end of the minimal enhancer. In fact, we have shown that removal of both E3 and E3′ results in almost complete loss of enhancer function (J. H. Patterson and P. A. Benfield, unpublished observations). The *ckb* TARP-binding site can substitute for E3 to restore enhancer func-

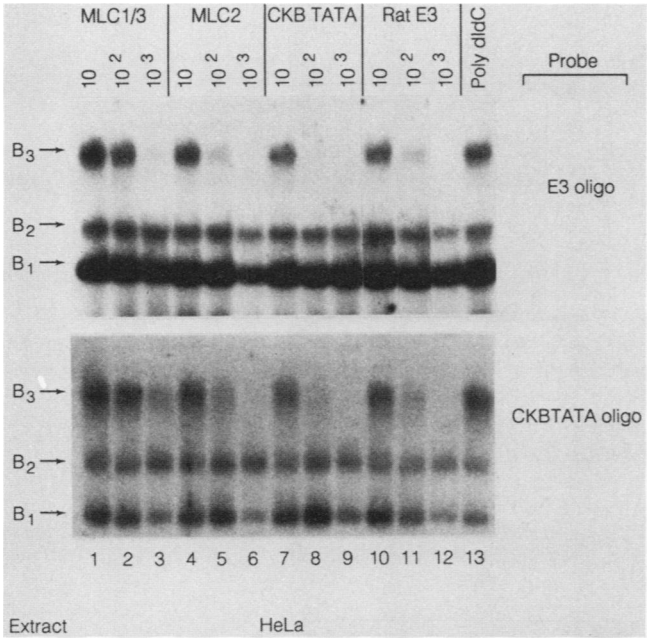

FIG. 4. Gel retardation assay performed by using [^{32}P]kinase end-labeled synthetic oligonucleotides E3 and CKBTATA as indicated. The experiment was performed by using nuclear extracts from HeLa cells as shown at the bottom. Incubations were carried out in the presence of 3 μg of nonspecific poly(dI-dC) alone or together with increasing concentrations of cold oligonucleotides LC1/3 (MLC1/3), LC2 (MLC2), *ckb* TATA (CKB TATA), and rat E3 as shown at the top. Only bands retarded in the gel are shown. Bands B_1 and B_2 appear to be derived from nonspecific binding to the probe. Band B_3 is derived from specific binding to the oligonucleotide probes.

tion. Thus, in the *ckm* enhancer, TARP-binding sites contribute to expression in muscle.

The observation that removal of the E3 regions from the rat *ckm* enhancer leads to a dramatic loss in enhancer activity suggests that the remaining myoD-binding sites are alone insufficient to generate full muscle-specific enhancer activity. Rather, proteins of the myoD family may work in combination with other proteins that may not necessarily be muscle specific. TARP is a candidate for such a protein.

Alternatively, the enhancer may drive muscle-specific activation in response to several different *trans*-acting factors. The effect of deletion of TARP-binding sites in the *ckm* enhancer it less dramatic when larger segments of enhancer or promoter are tested (24; P. A. Benfield and R. Horlick, unpublished observations). Thus, other elements may be present in the enhancer-promoter region of this gene that can substitute for TARP. This latter observation may account for the variability in reports describing the effects of E3 deletion on *ckm* enhancer function. Buskin and Hauschka (9) observe a much less dramatic effect of E3 deletion on mouse *ckm* enhancer function. This difference may result from subtle differences in the enhancer constructs tested.

TARP-binding sites have been found in at least two other muscle-specific genes, the cardiac myosin LC2 gene promoter (1, 8) and the enhancer for the rat skeletal muscle LC1/3 gene (16). In the cardiac myosin LC2 gene, the TARP-binding sequence forms the distal promoter element shown to be essential for up regulation of this promoter during myogenesis (8). Furthermore, these authors report that the distal promoter element site alone can confer muscle specificity to the *tk* TATA box. The function of the sequence in the rat skeletal muscle LC1/3 enhancer remains to be determined. Although the *ckb* gene is not muscle specific, it is expressed along with *ckm* in adult cardiac muscle and also in smooth muscle (48). So far, we have not detected TARP-binding sites in any gene that is not expressed in at least one type of muscle. These observations suggest that TARP sites may play an important role in muscle gene regulation.

AACCCAGACATGTGGCTGCTCC	Mouse E1 (-)
CCTGGTTATAATTAACCTGGACACGTGGTTGCTCCC	Rat E1
TTAACCTGGACACGTGGTTGCTCCCCCC	Rat medium E1 (-)
CTGGACACGTGGTTGCTCCCC	Rat short E1 (-)
TGCCTGGTTATAATTAACCTGGA	Rat E3'
AAGCTTGCTCTAAAAATAACTCTATCCC	Rat E3
GAATGAATGGGCTATAAATAGCCGCCA	Rat *ckb* TARP
CCAGGCTAAAAATAACCCCATGTCCAC	Chicken cardiac MLC2
CATCTTTTAAAAATAACTTTTCAAA	Rat skeletal MLC 1/3
GCTAAAAATAACC TT T T G T	CONSENSUS
GAATGAATGCGCTTAAATTTGAGGCCA	Adeno EIIAe T2 (-)

FIG. 5. Sequences with and without TARP-binding sites. The sequences are presented so as to align similar sequences. The origin of each sequence is shown on the right. (−), Sequences that do not bind TARP. Data supporting the binding or lack of binding to mouse E1, rat mutant TARP, and adenovirus (adeno) EIIAe T2 are not shown. A consensus sequence for TARP binding has been derived and is shown in the second line from the bottom. The adenovirus EIIAe promoter shows striking homology to the rat and human *ckb* promoters (13, 23). The T2 sequences lies in the equivalent position in the adenovirus promoter to the *ckb* TATA sequence. However, it does not constitute a binding site for TARP, as judged by the gel retardation assay. MLC, Myosin light chain.

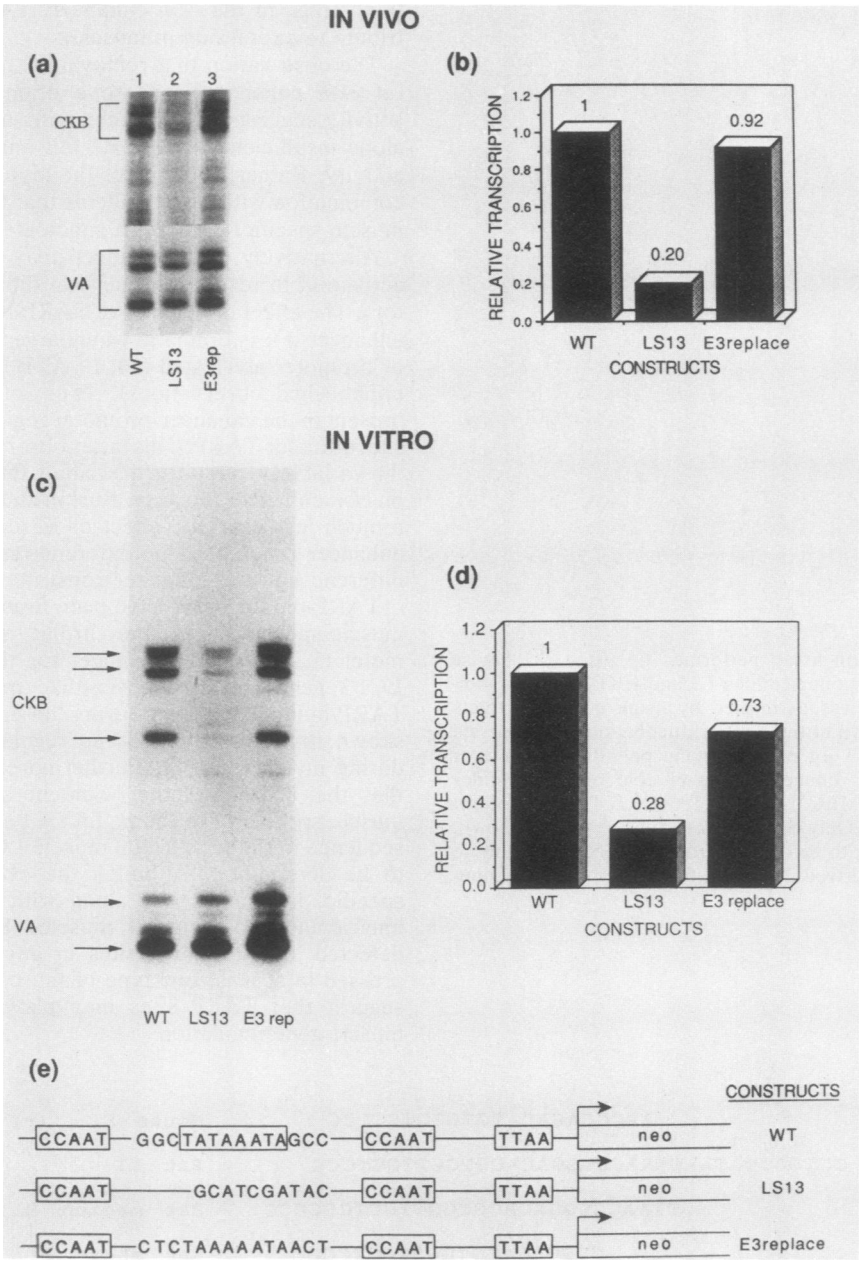

FIG. 6. Schematic representation of the rat *ckb* promoter *neo* constructs is shown in panel e. Major potential regulatory elements (CCAAT, TATAAATA, and TTAA) are boxed. The transcript start point is indicated by a bent arrow. Sequence changes made in the E3replace construct and linker scan mutant (LS13) are indicated. These constructs were tested in a transient transfection assay in HeLa cells as described in Materials and Methods. RNA was harvested, and *neo* and VA control RNAs were assayed by primer extension (a). The relative transcription of the *neo* gene in different mutant constructs with respect to wild type was determined as test *neo*/test VA divided by wild-type (WT) *neo*/wild-type VA (b). The constructs (e) were tested by in vitro transcription using HeLa cell nuclear extracts. RNA generated was analyzed by primer extension (c). The migration positions of products derived from *ckb* promoter-driven *neo* transcripts (CKB) and VA RNA (VA) are indicated by arrows on the left. (d) Relative transcription of the *neo* gene relative to that of the VA control; Results are averages of two experiments. In vivo results for LS13 varied between 0.2 and 0.5, and those for E3replace varied between 0.92 and 1.5. However, the two results varied in parallel. Similarly, the in vitro results for LS13 varied between 0.16 and 0.4 and those for E3replace varied between 0.57 and 0.9, again in parallel.

However, TARP-binding sites also function in nonmuscle cells. The *ckb* promoter is active in HeLa cells, and mutation of the TARP-binding site leads to a diminution of promoter activity. Again, the TARP-binding site from the *ckm* enhancer can substitute for upstream TATA element in this promoter to restore complete promoter activity.

The positive action of TARP-binding sites can be demonstrated in vitro with HeLa cell nuclear extracts, suggesting that TARP may be a positively acting transcription factor. Like many transcription factors, it appears to be able to function in both a promoter and an enhancer context (29, 45, 46).

The TARP-binding site has an AT-rich core, although mutations in this core are tolerated. However, TARP does

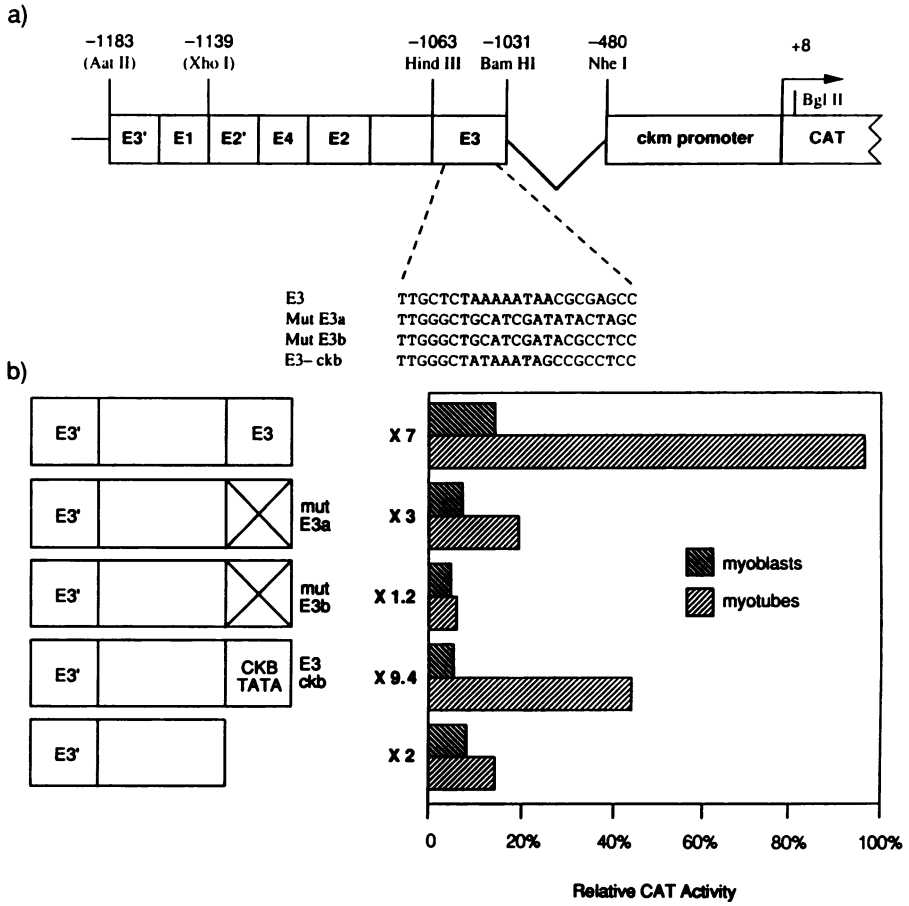

FIG. 7. (a) Construction of mutants in the rat 5′ *ckm* enhancer. *ckm* promoter-enhancer CAT constructs were generated that contained the following elements. The *ckm* promoter from the *Nhe*I site at −480 to a synthetic *Bgl*II site at +8 was linked to the CAT gene. Upstream was placed the minimal 5′ *ckm* enhancer from the *Bam*HI site at −1031 to a synthetic *Aat*II at −1183. This enhancer also contains a synthetic *Xho*I site at −1139 (see Materials and Methods). The sequence of the wild-type D3 region contained in this construct is shown at the bottom. Also shown are the sequences of two mutants, Mut E3a and Mut E3b, and of a third mutant that replaces the E3 region with the upstream TATA region of the rat *ckb* promoter (E3-ckb). The transcript start point is indicated by a bent arrow. (b) Results of gene transfer experiments using the constructs depicted in panel a. Each construct is shown schematically on the left. The region shown diagrams the enhancer region in each of these constructs. The E3 and E3′ regions are boxed and shown separately. Mutations in the E3 region are shown. A further mutant in which the entire E3 region is removed was also tested. This mutant was derived by cleaving plasmid Xba 1.5 CAT with *Hin*dIII (−1063) and *Nhe*I (−480) and religation after blunt ending with DNA polymerase. CAT activities are shown as arbitrary units. Induction ratios varied from 5 to 10 for the wild-type enhancer, from 1 to 3 for each of the mutant enhancers, and from 9.4 to 12 for the CKBTATA replace construct. The results shown are from a single transfection.

not bind to AT-rich sequences in general or to all TATA boxes (Benfield, unpublished observations). Rather, sequences outside the AT-rich region appear to be important for binding. In this respect, its binding specificity appears to differ from that of previously described AT-rich-binding proteins (42, 45). A C residue at the 3′ end of the consensus sequence is invariant in all the TARP sequences shown in Fig. 5, although it is changed to a G in the equivalent sequence from chicken *ckb* (T. Wirz and J.-C. Perriard, personal communication). Similarly, a C residue at the 5′ end of the AT-rich core is invariant in all of the strong TARP-binding sequences.

Another AT-rich sequence that appears to be important for muscle gene regulation is the CArG box. This sequence binds a factor that is probably identical to the serum response factor (6). A second muscle-specific CArG box-binding factor has also been described (49, 50). Binding of TARP is not competed for by any functional CArG box (Fig. 3; R. Horlick, unpublished observations). Thus, TARP is distinct from the factors that recognize CArG-related sequences.

TARP-binding sites appear to contribute to muscle specificity. Gossett and co-workers (19) have described two factors, mbf1 and mef2, that bind the E3 region of the mouse *ckm* enhancer. These give rise to different mobilities in gel retardation assays. The factor mbf1 is found most prominently in myoblasts, and mef2 appears to be myotube specific. Since TARP is not muscle specific, it appears to be different from either of these two activities. It is possible that the activity of the AT-rich segment in the *ckm* enhancer results from a muscle-specific activity such as mef2. However, our evidence strongly indicates that binding to the TARP sequence is not a muscle-specific phenomenon and furthermore that factors that recognize this sequence, possibly TARP, are functional in nonmuscle cells. This latter observation is of interest given the recent reports that the *ckm* gene may be expressed in sources other than muscle cells, e.g., brain (21, 27). Although the locations of se-

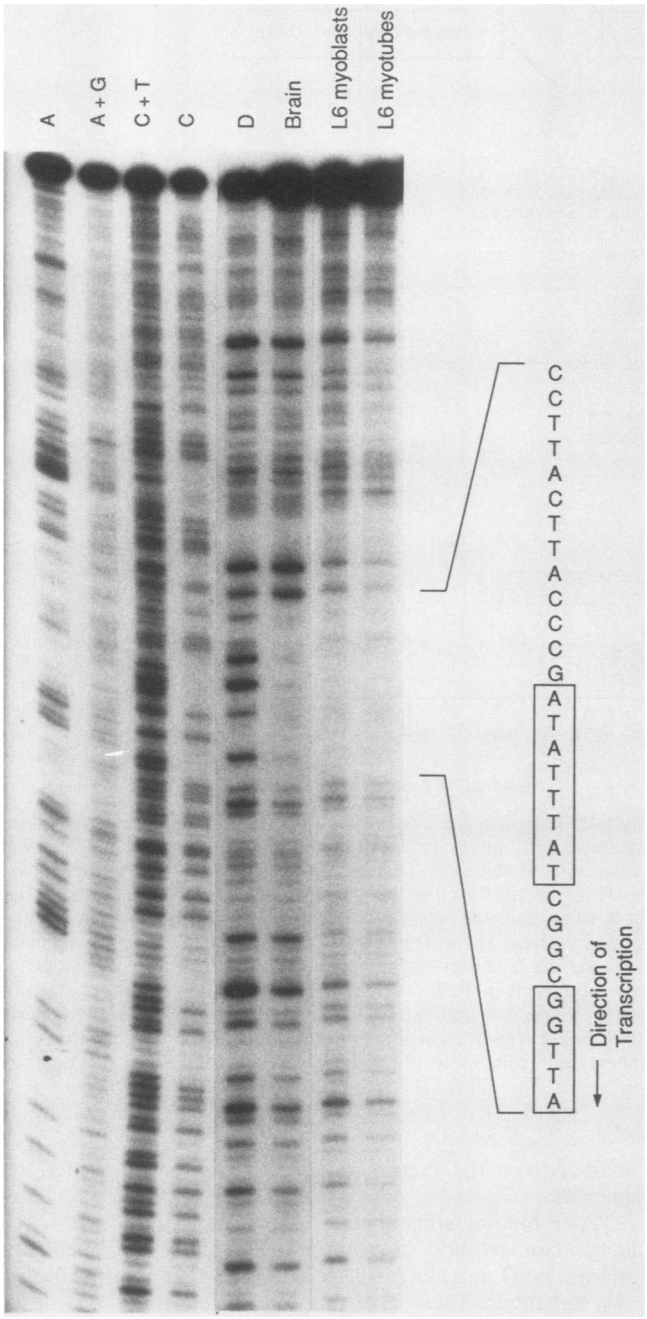

FIG. 8. Results of DNase I footprinting on the *ckb* promoter, using nuclear extracts derived from brain tissue, L6 myoblasts, and L6 myotubes. The probe used was a *Hin*fI-*Pst*I fragment from the rat *ckb* promoter end labeled on the noncoding strand with polynucleotide kinase (see Materials and Methods). The sequence in the area of protection was determined from marker sequencing lanes (12, 31) and is shown on the right together with an arrow that indicates the direction of transcription. The upstream TATA region and the downstream CCAAT box are boxed. Lane D, Digestion with DNase I in the absence of nuclear extract.

quences responsible for this expression are not known, it is interesting that the *ckm* and *ckb* genes contain a common regulatory element.

The presence of two binding sites for TARP within the minimal enhancer is of interest. Several protein-binding sites are duplicated within the minimal enhancer segment. Two binding sites for myoD are found in this enhancer (28). These sites are presumably crucial to muscle-specific regulation of the *ckm* gene, and it has been shown that mutation of the right myoD (mef1)-binding site leads to a dramatic loss of enhancer function (9). Like the TARP-binding sites, these sites do not bind myoD with equal affinity (28). Also, a protein that binds A GC-rich sequence (E2) has been identified (24). This protein also has two binding sites within the *ckm* enhancer. Finally, a protein that recognizes the sequence TGCCTGG in the acetylcholine receptor (ACR) δ subunit promoter has been described (3). A related binding sequence (ACRE) is also present twice in the *ckm* minimal enhancer segment. In the ACR gene, the *cis*-acting element responsible for muscle-specific up regulation maps to a reiterated array of this sequence rather than to a nearby myoD-binding site (3). A summary of potential protein-binding sites in the *ckm* enhancer is presented in Fig. 9. Recently, Mueller and Wold (35) have used in vivo footprinting on the mouse *ckm* enhancer to show that the enhancer appears to be unoccupied before myotube formation. Upon differentiation, several sites in the enhancer become protected (Fig. 9). Included in these sites is the 3′ E3 TARP-binding site. This finding further supports the importance of this site in *ckm* muscle-specific enhancer function. It is of interest that the E1-binding protein recognizes a sequence that overlaps the left myoD-binding site (28). However, it is presumably distinct from myoD in that it is not muscle specific.

The significance of the duplication of these sites is uncertain. Duplicated myoD-binding sites are associated with many muscle-specific genes. However, we have shown for the rat *ckm* enhancer that removal of the left myoD-binding site results in a fully functional enhancer (Patterson and Benfield, unpublished data). Therefore, unless there is another myoD-binding site as yet undetected in this region, two myoD-binding sites would appear not to be necessary for muscle-specific enhancer function.

In conclusion, we have shown that TARP recognizes sequences in the *ckm* enhancer and *ckb* promoter that are important for regulated expression of these two genes. The TARP recognition sequence is functional in nonmuscle cells but contributes to expression in muscle cells. Since TARP activity appears to be ubiquitously present, it is suggested that its action on transcription depends on the context in which binding sites are found. In the context of the *ckm* enhancer, it contributes to muscle-specific transcription. This may be similar to the situation that has been described for κB elements that can function synergistically with NF1 elements to activate gene expression in nonlymphoid cells (22). However, in the context of the *ckb* promoter, in combination with CCAAT sequences, TARP elements are functional in nonmuscle cells. Alternately, a second activity is present that is muscle specific and interacts with the same TARP-binding site to generate muscle-specific expression. In the latter scenario, a mechanism would have to exist to prevent activation in nonmuscle cells. This situation would then be analogous to the situation existing for genes containing octamer-binding sites that are differentially activated by the tissue-specific and cell-specific octamer-binding factors (29, 45, 46).

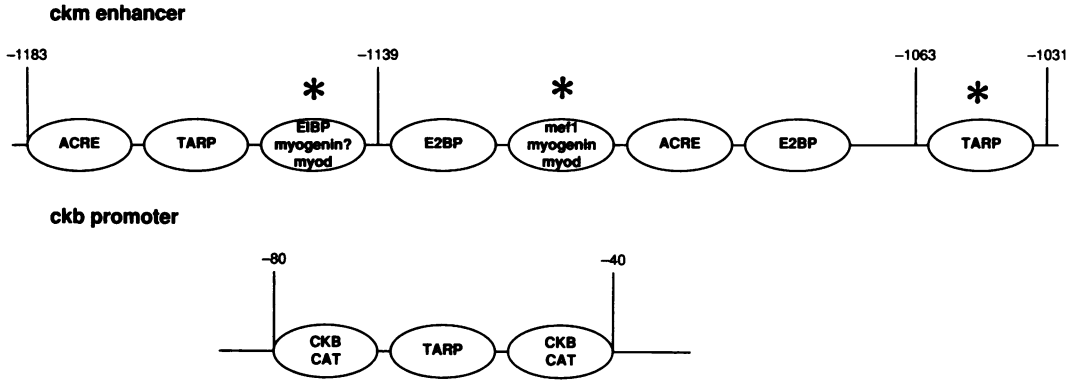

FIG. 9. Summary of possible binding activities that have been described to recognize the *ckm* enhancer or the *ckb* promoter. The approximate locations of binding sites are indicated by the numbers. Proposed binding activities are enclosed in ellipses. ACRE refers to a muscle-specific binding activity that has been described to recognize a similar element in the acetylcholine receptor, although binding to the *ckm* enhancer has not been demonstrated. This element in the acetylcholine receptor gene has been shown to be necessary for muscle-specific activation of this gene, E1BP refers to a protein that recognizes the core E1 region. E2BP recognizes a GC-rich region that occurs twice in the enhancer. CKB CAT represents a CCAAT-binding activity that that recognizes the CCAAT sequences in the *ckb* promoter. Asterisks indicate regions shown by in vivo footprinting analysis to be protected (35).

ACKNOWLEDGMENTS

We thank Blair Ferguson and Gerald Jonak for critical reading of the manuscript and Mark Pearson for his constant encouragement and support.

ADDENDUM IN PROOF

While this manuscript was in press, a correction for reference 3 was published. The correction [Nature (London) **345**:364, 1990] indicated that although the ACRE element is necessary for muscle-specific regulation of the murine acetylcholine receptor σ subunit promoter, there is no evidence that the *cis*-acting regulatory region described that lacks a MyoD1-binding site (−148 to −95) is sufficient to confer muscle-specific expression.

LITERATURE CITED

1. **Arnold, H. H., E. Tannich, and B. M. Paterson.** 1988. The promoter of the chicken cardiac myosin light chain 2 gene shows cell-specific expression in transfected primary cultures of chicken muscle. Nucleic Acids Res. **16**:2411–2429.
2. **Babiss, L. E., R. S. Herbst, A. L. Bennett, and J. E. Darnell, Jr.** 1987. Factors that interact with the rat albumin promoter are present both in hepatocytes and other cell types. Genes Dev. **1**:256–267.
3. **Baldwin, T. J., and S. J. Burden.** 1989. Muscle-specific gene expression controlled by a regulatory element lacking a myoD1 binding site. Nature (London) **341**:716–720.
4. **Benfield, P. A., D. Graf, P. N. Korolkoff, G. Hobson, and M. L. Pearson.** 1988. Isolation of four rat creatine kinase genes and identification of multiple promoter sequences within the rat brain creatine kinase promoter. Gene **63**:227–243.
5. **Bond-Matthews, B., and N. Davidson.** 1988. Transcription from each of the *Drosophila act 5c* leader exons is driven by a separate functional promoter. Gene **62**:289–300.
6. **Boxer, L. M., R. Prywes, R. G. Roeder, and L. Kedes.** 1989. The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response factor. Mol. Cell. Biol. **9**:515–522.
7. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
8. **Braun, T., E. Tannich, G. Buschhausen-Denker, and H.-H. Arnold.** 1989. Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with *trans*-acting regulatory factors for muscle-specific transcription. Mol. Cell. Biol. **9**:2513–2525.
9. **Buskin, J. N., and S. D. Hauschka.** 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell. Biol. **9**:2627–2640.
10. **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.
11. **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.
12. **Chuvpilo, S. A., and V. V. Kravchenko.** 1984. A simple and rapid method for sequencing DNA. FEBS Lett. **179**:34–36.
13. **Daouk, G. H., R. Kaddurah-Daouk, S. Putney, R. Kingston, and P. Schimmel.** 1988. Isolation of a functional human gene for grain creatine kinase. J. Biol. Chem. **263**:2442–2446.
14. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
15. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
16. **Donoghue, M., H. Ernst, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal.** 1988. A muscle-specific enhancer is located at the 3′ end of the myosin 1/3 gene locus. Genes Dev. **2**:1779–1790.
17. **Edmondson, D. G., and E. N. Olson.** 1989. A gene with homology to the *myc* similarity region of myoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3**:628–640.
18. **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.
19. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. **9**:5022–5033.
20. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:539–549.
21. **Hamburg, R. J., D. L. Friedman, E. N. Olson, T. S. Ma, M. D. Cortez, C. Goodman, P. R. Puleo, and M. B. Perryman.** 1990. Muscle creatine kinase isoenzyme expression in adult human brain. J. Biol. Chem. **265**:6403–6409.
22. **Henninghausen, L., P. A. Furth, and C. W. Pittius.** 1989. κB

elements strongly activate gene expression in non-lymphoid cells and function synergistically with NF1 elements. Nucleic Acids Res. **17**:8197–8206.

23. **Hobson, G. M., M. T. Mitchell, G. R. Molloy, M. L. Pearson, and P. A. Benfield.** 1988. Identification of a novel TA-rich DNA binding protein that recognizes a TATA sequence within the brain creatine kinase promoter. Nucleic Acids Res. **16**:8925–8945.
24. **Horlick, R. A., and P. A. Benfield.** 1989. The upstream muscle-specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol. Cell. Biol. **9**:2396–2413.
25. **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 myoblast. Mol. Cell. Biol. **6**:2855–2864.
26. **Jaynes, J. B., J. E. Johnson, J. N. Buskin, C. L. Gartside, and S. D. Hauschka.** 1988. The muscle creatine kinase gene is regulated by multiple upstream elements including a muscle-specific enhancer. Mol. Cell. Biol. **8**:62–70.
27. **Johnson, J. E., B. J. Wold, and S. D. Hauschka.** 1989. Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. Mol. Cell. Biol. **9**:3393–3399.
28. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
29. **LeBowitz, J. H., T. Kobayashi, L. Staudt, D. Baltimore, and P. A. Sharp.** 1988. Octamer-binding proteins from B or HeLa cells stimulate transcription of the immunoglobulin heavy-chain promoter in vitro. Genes Dev. **2**:1227–1237.
30. **Lin, Z., C. A. Dechesne, J. Eldridge, and B. M. Paterson.** 1989. An avian muscle factor related to myoD1 activates muscle-specific promoters in non muscle cells of different germ-layer origin and in BrdU-treated myoblasts. Genes Dev. **3**:986–996.
31. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
32. **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 repeat motif. Mol. Cell. Biol. **6**:2125–2136.
33. **Mitchell, M. T., and P. A. Benfield.** 1990. Two different RNA polymerase II initiation complexes can assemble on the rat brain creatine kinase promoter. J. Biol. Chem. **265**:8259–8267.
34. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human cardiac α-actin gene. Mol. Cell. Biol. **7**:2803–2813.
35. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246**:780–785.
36. **Murre, C. P., P. Schonleber McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, MyoD and *myc* proteins. Cell **56**:777–783.
37. **Murre, C. P., P. Schonleber McCaw, H. Vassein, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.
38. **Perriard, J.-C.** 1979. Developmental regulation of creatine kinase isoenzymes in myogenic cell culture from chicken. J. Biol. Chem. **254**:7036–7041.
39. **Pinney, D. F., S. H. Pearson-White, S. F. Konieczny, K. E. Latham, and C. P. Emerson, Jr.** 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell **53**:781–793.
40. **Reinberg, D., M. Horikoshi, and R. G. Roeder.** 1987. Factors involved in specific transcription in mammalian RNA polymerase II. Functional analysis of initiation factors IIA and IID and identification of a new factor operating at sequences downstream of the initiation site. J. Biol. Chem. **262**:3322–3330.
41. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory gene family. Genes Dev. **3**:2050–2061.
42. **Sawadogo, M., and R. G. Roeder.** 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA **82**:4394–4398.
43. **Shapiro, D. J., P. A. Sharp, W. W. Wahl, and M. J. Keller.** 1988. A high efficiency HeLa nuclear transcription extract. DNA **7**:47–55.
44. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–158.
45. **Solomon, M. J., F. Strauss, and A. Varshavsky.** 1986. A mammalian high mobility group protein recognizes any stretch of six A-T base pairs in duplex DNA. Proc. Natl. Acad. Sci. USA **83**:1276–1280.
46. **Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore.** 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. Nature (London) **323**:640–643.
47. **Sternberg, E. A., G. Spizz, W. M. Perry, D. Vizard, T. Weil, and E. A. Olson.** 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. **8**:2896–2909.
48. **Urdal, P., K. Urdal, and J. H. Strømme.** 1983. Cytoplasmic creatine kinase isoenzymes quantitated in tissue specimens obtained at surgery. Clin. Chem. **29**:310–313.
49. **Walsh, K., and P. Schimmel.** 1987. Two nuclear factors compete for the skeletal muscle actin promoter. J. Biol. Chem. **262**:9429–9432.
50. **Walsh, K., and P. Schimmel.** 1988. DNA-binding site for two skeletal actin promoter factors is important for expression in muscle cells. Mol. Cell. Biol. **8**:1800–1802.
51. **Watts, D. C.** 1973. Creatine kinase (adenosine 5′-triphosphate-creatine phosphotransferase), p. 383–455. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. 8. Academic Press, Inc., New York.
52. **Weintraub, H., S. J. Tapscott, R. L. Davis, M. J. Thayer, M. A. Adam, A. B. Lassar, and D. A. Miller.** 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA **86**:5434–5438.
53. **Wigler, M. A., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single copy eukaryotic genes using total cellular DNA as a donor. Cell **14**:725–731.
54. **Wright, W. E., D. A. Sassoon, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis has a domain homologous to MyoD. Cell **56**:607–617.
55. **Yaffee, D., and O. Saxel.** 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature (London) **270**:725–727.