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Dual Tandem Promoter Elements Containing CCAC-Like Motifs From the Tetrodotoxin-Resistant Voltage-Sensitive Na^+ Channel (rSkM2) Gene Can Independently Drive Muscle-Specific Transcription in L6 Cells

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cis-Elements in the −129/+124 promoter segment of the rat tetrodotoxin-resistant voltage-gated sodium channel (rSkM2) gene that are responsible for reporter gene expression in cultured muscle cells were identified by deletion and scanning mutations. Nested 5′ deletion constructs, assayed in L6 myotubes and NIH3T3 cells, revealed that the minimum promoter allowing muscle-specific expression is contained within the −57 to +1 segment relative to the major transcription initiation site. In the context of the −129/+1 construct, however, scanning mutations in the −69/+1 segment failed to identify any critical promoter elements. In contrast, identical mutations in a minimal promoter (−57/+124) showed that all regions except −29/−20 are essential for expression, especially the −57/−40 segment, consistent with the 5′ deletion analysis. Further experiments showed that the distal (−129/−58) and proximal promoter (−57/+1) elements can independently drive reporter expression in L6 myotubes, but not in NIH3T3 fibroblasts. This pair of elements is similar in sequence and contains Sp1 sites (CCGCCC), CCAC-like motifs, but no E-boxes or MEF-2 sites. The two segments form similarly migrating complexes with L6 myotube nuclear extracts in gel-shift assays. Critical elements within the distal promoter element were defined by 10 base pair scanning mutations in the −119 to −60 region in the context of the −129/+1 segment containing a mutated −59/−50 segment that inactivates the proximal promoter. Nucleotides in the −119/−90 region, especially −109/−100, were the most important regions for distal promoter function. We conclude that the −129/+1 segment contains two tandem promoter elements, each of which can independently drive muscle-specific transcription. Supershifts with antibodies to Sp1 and myocyte nuclear factor (MNF) implicate the involvement of Sp1, MNF, and other novel factors in the transcriptional regulation of rSkM2 gene expression.

Sodium channel Skeletal muscle Expression *cis*-Element L6 myotubes
Electrophoresis mobility shift analysis

VOLTAGE-DEPENDENT activation of sodium channels is responsible for the upstroke of action potentials in nerve, skeletal muscle, and heart (2,14,21,26). The presence of sodium channel proteins has been documented across the animal kingdom including invertebrates and vertebrates, and these proteins are encoded by multigene families in mammalian species such as rodents and humans. Many different channel isoforms can be distinguished based on tissue distribution, developmental expression pattern, immuno-

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logical and electrophysiological characteristics, and relative resistance to toxins such as saxitoxin, tetrodotoxin (TTX), and μ-conotoxin (7,26,27,30,48,51, 55).

Skeletal muscle expresses two different voltage-dependent sodium channels—TTX-sensitive rSkM1 and TTX-resistant rSkM2 isoforms—which have been characterized electrophysiologically and pharmacologically (27,48,51). The human genes for these isoforms, SCN4A and SCN5A, and other vertebrate homologs have been cloned and sequenced.

Steady-state mRNA levels of SkM1 and SkM2 are regulated quite differently in skeletal muscle and heart during development, and in skeletal muscle following denervation. rSkM2 mRNA is expressed in immature or denervated skeletal muscle and adult heart, while rSkM1 mRNA is present only in mature skeletal muscle (27). rSkM2 mRNA expression dominates in the prenatal stage of skeletal muscle development (Zhang, unpublished observations), but decreases rapidly to undetectable levels in skeletal muscle immediately postpartum (27,53). The decrease in rSkM2 steady-state mRNA levels following birth is accompanied by dramatic increases in the level of rSkM1 mRNA. rSkM2 mRNA is not detectable in innervated adult skeletal muscle, but there is a striking induction of rSkM2 transcription within 48 h following denervation, while the level of rSkM1 mRNA remains nearly constant (27,53). Increases in rSkM2 mRNA expression are also observed in rat skeletal muscle cells when differentiating in primary culture, and in the rat myogenic cell line L6 during the transition from replicating myoblasts to terminally differentiated multinucleated myotubes (43).

During differentiation, the temporal and spatial pattern of rSkM2 expression is distinct not only from that of rSkM1 but from many other sarcomeric proteins. This suggests that rSkM2 transcription may be controlled, at least in part, by factors different from those involved in expression of rSkM1 and other classical markers of terminally differentiated muscle cells. Consistent with current ideas, we anticipated that channel isoform gene expression would depend on basal, tissue- and stage-specific transcription factors interacting with the core promoter and additional upstream/downstream positive and negative elements.

We previously identified and partially characterized 5′-flanking regions of the rSkM1 and rSkM2 genes and showed that these fragments contain the transcription initiation region, core promoter, and other positive and negative controlling elements (29,43). The two muscle Na^+ channel genes have TATA-less promoters, contain E-boxes and GC-rich sequences. rSkM1 is positively and negatively controlled by two E-boxes in the promoter (29). A rSkM2 construct with several upstream positive and negative elements, when combined with the core promoter, gives the same level of transcriptional activity in the L6 muscle cell model system as does the core promoter (−129/+124) and also shows increased levels of expression in cells that have progressed into myotubes from myoblasts (43). However, within the −129/+124 region of the rSkM2 gene there are no E-boxes, suggesting that, unlike rSkM1, rSkM2 expression may be less dependent or even independent of E-box elements (43). The −129/+124 region does contain four consensus Sp1 binding sites (CCGCCC) (12) and multiple CCAC-like motifs (CCCCACCCC) (3,4,23). CCAC-box *cis*-elements have been reported to bind myocyte nuclear factor (MNF) (5) and appear to be key controlling elements within human myoglobin and other muscle-specific genes (Table 1). However, the function of these potential transcription factor recognition sites, and other *cis*-elements within the rSkM2 core promoter, have not been evaluated previously.

To remedy this situation, we turned to transient transfection studies of promoter constructs driving reporter genes in L6 cells, as well as to gel-shift analyses to characterize the *cis*-elements. The structure of the −129/+1 segment in the rSkM2 promoter has been examined in detail by deletion or substitution mutation analysis, and the results lead us to conclude that the rSkM2 promoter contains dual tandem elements, either of which can drive transcription. In addition, supershifts with antibodies to Sp1 or MNF implicate the involvement of Sp1 and MNF, as well as other novel transcription factor(s), in rSkM2 expression in muscle cells.

MATERIALS AND METHODS

Materials

Chemicals of the highest grade available were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). Mutagenic oligonucleotides, Lipofectamine™, DMEM and Opti-MEM™ media, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). L6 and NIH3T3 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Sp1 and retinoblastoma antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MNF and htβ antibodies and MNF-α and MNF-β genes were generous gifts from Drs. Rhonda Bassel-Duby and R. Sanders Williams (University of Texas Southwestern Medical Center, Dallas, TX).

Plasmid Constructions and hH1 Promoter Sequence

5′ Deletion and base substitution mutations were made in the *Hin*dIII/*Pst*I −129/+124 segment of the rSkM2 gene as previously described (43) except that the pALTER1 mutagenesis system from Promega (Madison, WI) was used following the manufacturer's instructions. The recombinant DNA molecules were analyzed by restriction enzyme digestion and the −129/+124 promoter regions were verified by sequencing with ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). The 5′-flanking region of the human homologue of rSkM2, hH1, contained in a 6.6-kb genomic segment, generously contributed by Dr. Alfred L. George, Jr., was sequenced as described above.

Cell Culture and Transfection. The effect on function of each mutation in the rSkM2 promoter was examined by transfection of the constructs using Lipofectamine™ into L6 or NIH3T3 cells with Opti-MEM™ medium following manufacturer's instruction (43,57). Muscle cell differentiation was carried out as described elsewhere (43,57).

CAT and β-Galactosidase Activity Assays

The activities of the promoters were measured by the relative expression levels of CAT and β-galactosidase in transfected cells as described (43,57).

Preparation of Nuclear Extracts and Gel-Shift Assay

Nuclear factors in extracts from L6 myotubes or NIH3T3 cells (18) that bind to the promoter region were analyzed by gel-shift assays as described elsewhere (43,57).

UV Cross-Linking

The binding reactions were carried out as described elsewhere (43,57), but in a total volume of 50 μl containing 5 pmol (2×10^5 cpm) of labeled probe, 27 μg of nuclear extract protein. The UV cross-linking was carried out in a 1.5-ml round-bottom vial sealed with plastic wrap and irradiated from overhead with a Mineralight Lamp (UVGL-25, UVP Inc., San Gabriel CA, 254 nm wavelength) at a distance of 5 cm for 3 h at 4°C. After cross-linking, 50 μl of 2× sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 30 mM DTT, with a few crystals of bromophenol blue) was added to each sample and incubated at 95°C for 5 min. The proteins were resolved on a 12% SDS-polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) electrophoresis at 100 mV at 4°C and, after drying, visualized (57).

Analysis of Data

Estimates of promoter activity obtained from transfection experiments, with two or more DNA preparations in triplicate or greater, are presented as mean ± SD (57). The Student's *t*-test was used to determine the statistical significance of the experimental vs. control plasmids.

RESULTS

Deletion Analysis of the −129/+1 Promoter Segment Demonstrates That the −57/−50 Region Is Essential for rSkM2 Promoter Activity in Muscle Cells

L6 and NIH3T3 cells were transfected with the CAT reporter gene driven by a series of 5′ deletions within the −129/+1 promoter region. Deletion of the +26 to +124 DNA segment has no effect on promoter activity (data not shown), and for convenience in construction, the mutant promoters retain the downstream segment to +124. A −129/-57 deletion to produce the −57/+124 promoter increases CAT expression by 1.8-fold compared to that of −129/+124, while further deletion to −49 decreases promoter activity in the L6 myotubes to ~13% of that of the −57/+124 construct (Fig. 1). The −57/+124 construct is the smallest promoter segment capable of driving high-level expression of CAT in L6 myotubes. Thus, in the absence of upstream elements, the region between −57 and −50 contains *cis*-elements that are important for rSkM2 expression in muscle cells. Consistent with this assignment, lower promoter activities were also detected with the −49/+124 and −39/+124 deletion constructs (Fig. 1), perhaps due to the removal of CCAC-like motifs. These constructs retain a Sp1 site in the −28/+7 region (Figs. 5 and 6).

The promoter region of the homologous human TTX-resistant sodium channel (hH1) was sequenced to search for evolutionarily conserved regions that might reflect functional importance. Comparison of the rSkM2 and hH1 sequences between −240 and +124 reveals ~97% identity in the region between −141 and −21. There is an apparent 11-bp insertion in the hH1 gene (at −57 of the rSkM2 sequence, Fig. 2) and in this region there are multiple consensus binding sites for Sp1, as well as several CCAC-like motifs. Sp1 has been recognized to be a general transcription factor whereas the CCAC-like motifs appear to be more specific, being involved in the expression of muscle-specific genes such as myoglobin (Table 1). The 11-bp insert in the hH1 promoter introduces an additional copy of a Sp1 site and a CCAC-like motif.

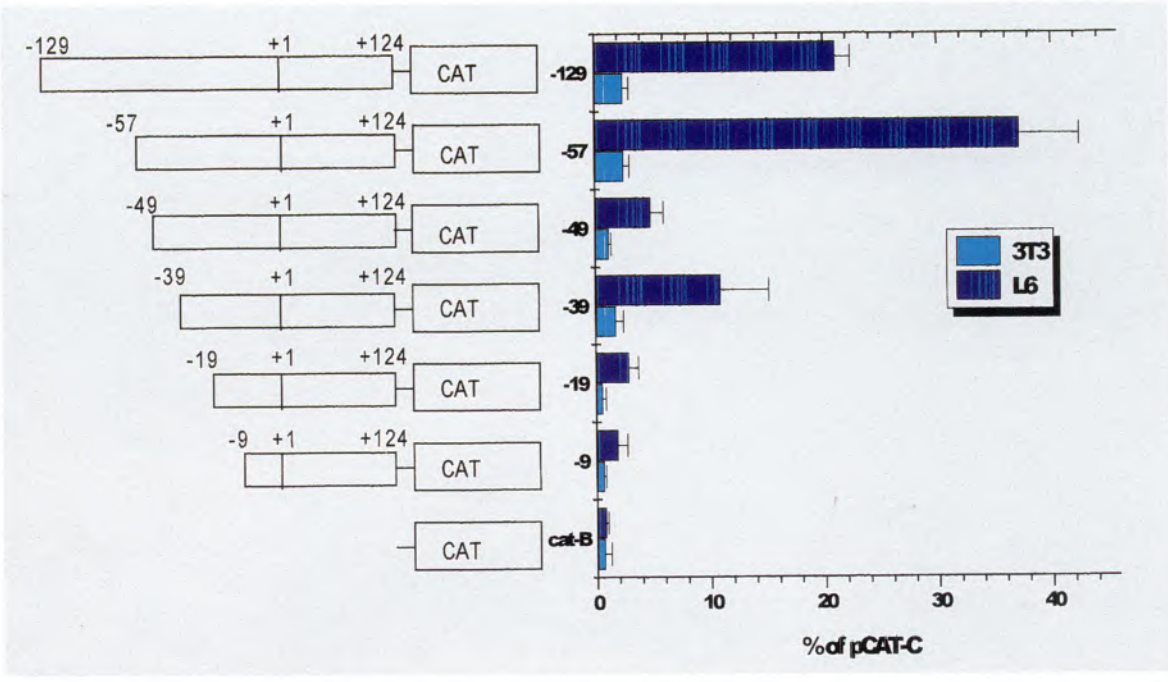


FIG. 1. Promoter activities of 5′ deletions in the rSkM2 promoter region. Relative CAT expression levels are stated as a percent of the activity of pCAT-C (normalized with β-galactosidase activities obtained from cotransfected RSV-β-galactosidase plasmids). Error bars are based on the average of at least three separate transient transfection experiments.

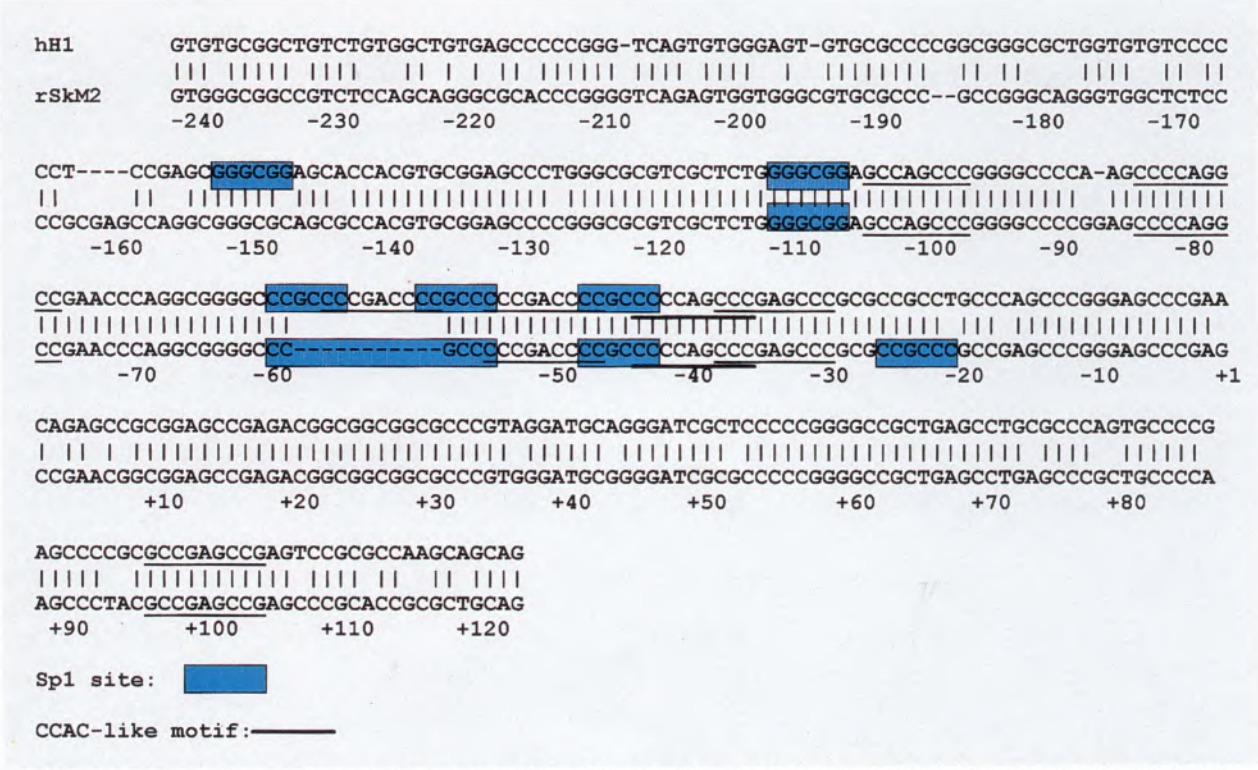


FIG. 2. Nucleotide sequence alignment of promoter regions of human and rat TTX-resistant sodium channel genes, hH1 and rSkM2. Nucleotides are numbered relative to the major transcription initiation site of rSkM2 (+1). The putative Sp1 binding sites are enclosed in boxes; the underlined sequences indicate CCAC-like motifs.

TABLE 1
CCAC-LIKE MOTIFS

Genes	*cis*-Elements	CCAC-Like Motifs	Locations	References
Mouse AChR δ subunit	−148 to −53	CCCCACCCC	−99 to −91	(1)
Chick AChR δ subunit	−207 to −146	CCCCAGCACC	−117 to −106	(49)
Human cardiac α-actin	−113 to −26	CCCCACCCC	−80 to −72	(41)
Human myoglobin	−261 to −205	CCCCACCCC	−219 to −211	(17)
β-MHC	−354 to −182	CCCCACCCC	−259 to −250	(47)
Mouse MCK	−1351 to −1050	CCCCACCCC	−1243 to −1121	(44)
rSkM2	−129 to +1	GCCAGCCC	−103 to −96	(43) and this work
		CCCCAGGCC	−83 to −75	
		CCCGACCCC	−54 to −46	
		CCCCAGCCC	−43 to −35	
		CCCGAGCCC	−37 to −29	

rSkM2 Core Promoter Consists of Dual Tandem Promoter Elements, Each of Which Drives Expression in L6 Myotubes

To identify the *cis*-elements in the −57/+1 region, scanning mutations through the −69/+1 region were created in the context of the −129/+124 segment (Fig. 3A). Surprisingly, none of the 10-bp block mutations in the −129/+124 context resulted in a substantial reduction in CAT gene expression (Fig. 3A). This is despite the fact that 5′ deletions extending to −50, associated with the removal of −57/−50 region, caused an almost complete loss of transcriptional activity (Fig. 1). How could block mutations of this same region in the −129/+124 context paradoxically have little effect (Fig. 3A)? One possibility is that *cis*-elements in the −129/−70 region function as a promoter in this in vitro system quite independent of the −57/+1 segment. In support of this explanation, a sequence alignment of the −129/−58 and −57/+1 segments reveals multiple similarities in the region between −110/−57 and −59/−10, including Sp1 consensus and CCAC-like motifs (Fig. 3B).

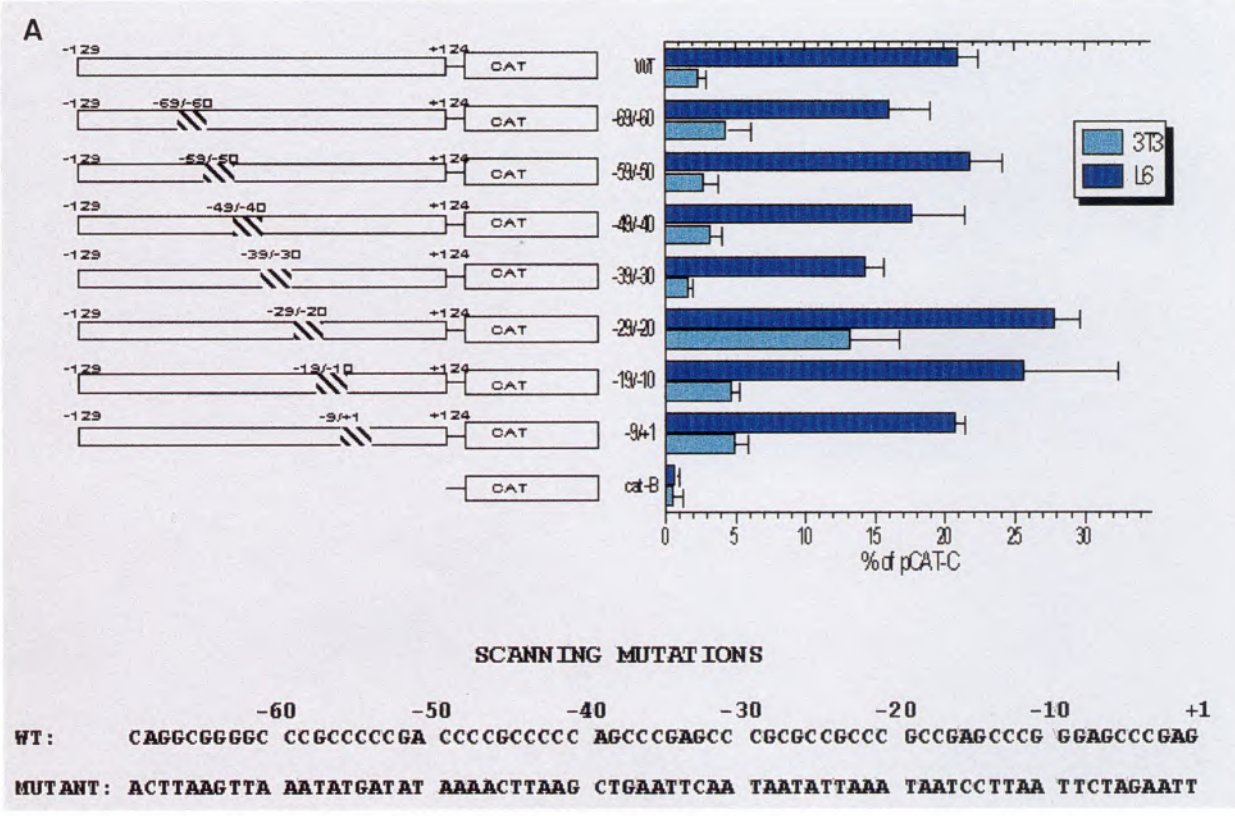
To further examine whether dual promoter elements exist, the scanning mutation analysis was repeated in the context of −57/+124 segment. In contrast to the results in the −129/+124 construct, dramatic reductions in CAT activity were found with mutations throughout this region, most prominently in the −57/−50 segment (Fig. 3C). The effects of these mutations were substantially more pronounced in L6 myotubes, further supporting the importance of the −57/−50 segment in controlling expression in differentiated muscle, presumably because −57/−50 recruits muscle-specific nuclear factors. We conclude that there are redundant elements located in distal and proximal portions of the −129/+1 promoter segment. Further evidence in support of this conclusion is that deletion constructs missing nucleotides −49 to −11 or −57 to −11 of the proximal segment still support muscle cell-specific transcription, at ~60% (Δ −49/−11) or ~40% (Δ −57/−11) of the activity expressed by the intact −129/+124 segment (Fig. 3D). These results provide strong support for the notion that the rSkM2 promoter is composed of distal (−129/−58) and proximal (−57/+1) promoter elements, each of which can support muscle-specific expression in vitro.

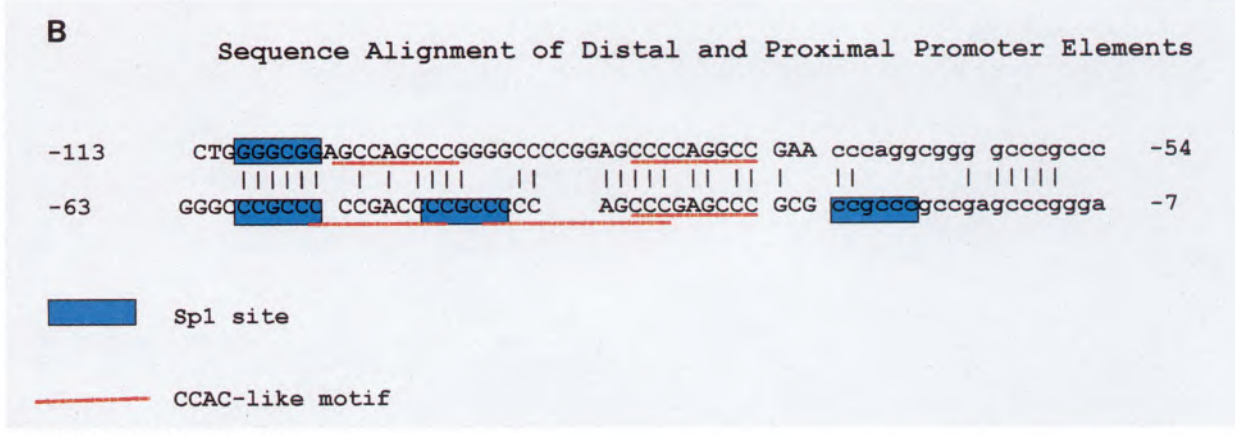
Nucleotides Located in the −119/−90 Region, Especially −109/−100, Are Critical for Distal Promoter Function

In order to identify critical elements in the distal promoter region, we analyzed the −129/+124 rSkM2 promoter containing the −59/−50 block mutation, which inactivates the proximal promoter (Fig. 3C). Scanning 10-bp block mutations between −119 and −60 identified −119/−90 as the most critical segment in the distal promoter region (Fig. 4) with the −109/−100 mutation decreasing promoter activity in L6 cells to background levels. These data support our conclusion that the rSkM2 promoter contains two promoter elements organized in tandem. Either region can sustain expression in the presence of deleterious mutations or deletions in the other segment. Mutations within the −109/−100 region remove a Sp1 site and an adjacent CCAC-like motif, corresponding approximately to mutations within the −57/−50 region of the proximal promoter element (Fig. 3B).

cis-*Elements in the Distal and Proximal Promoter Segments Bind Similar Sets of Nuclear Proteins Including Sp1*

If the distal and proximal promoter elements are functionally equivalent, then they might be expected to bind similar sets of transcription factors. This possibility was tested by gel-shift experiments. DNA

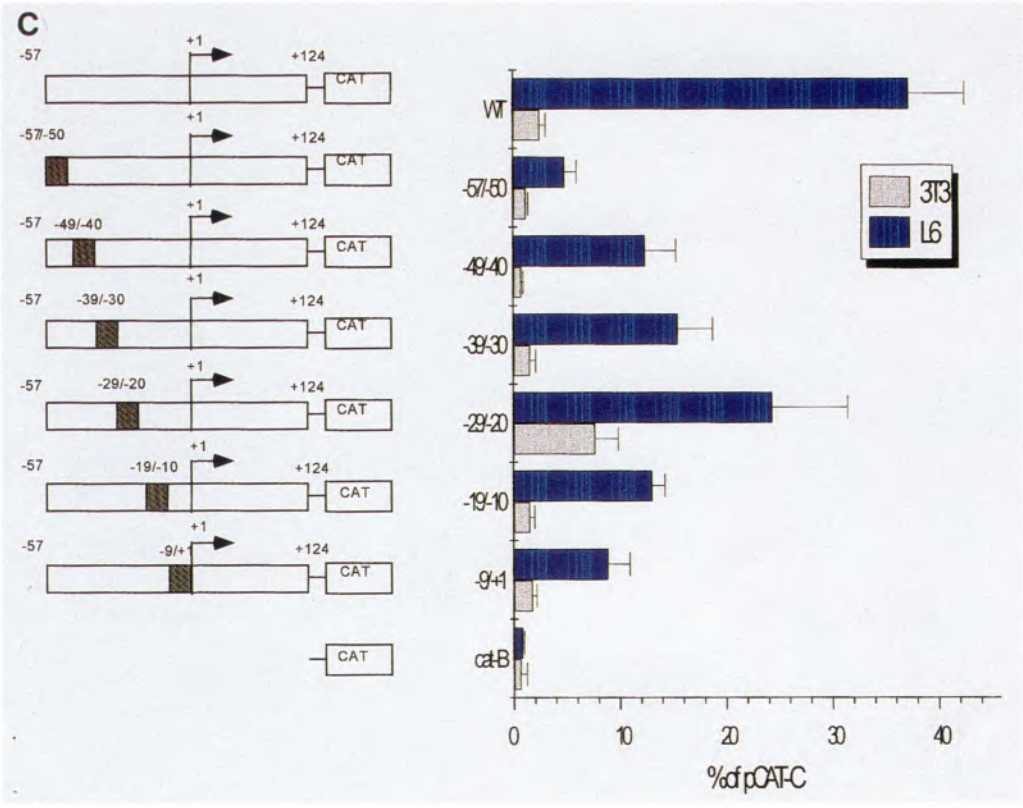


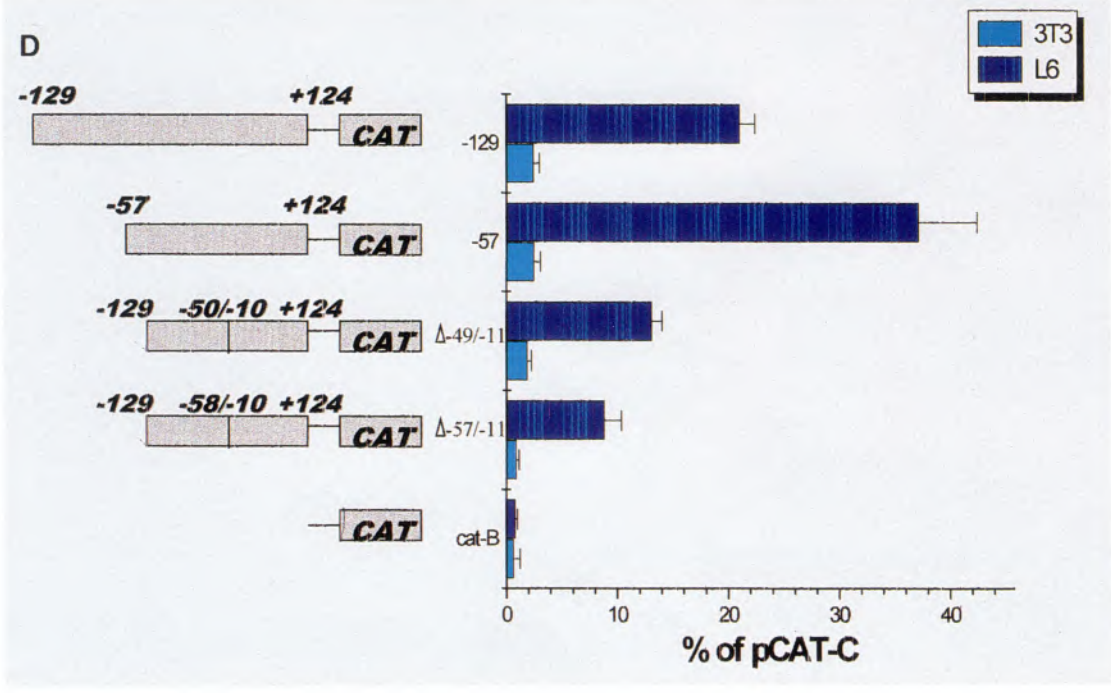


ABOVE AND FACING PAGE

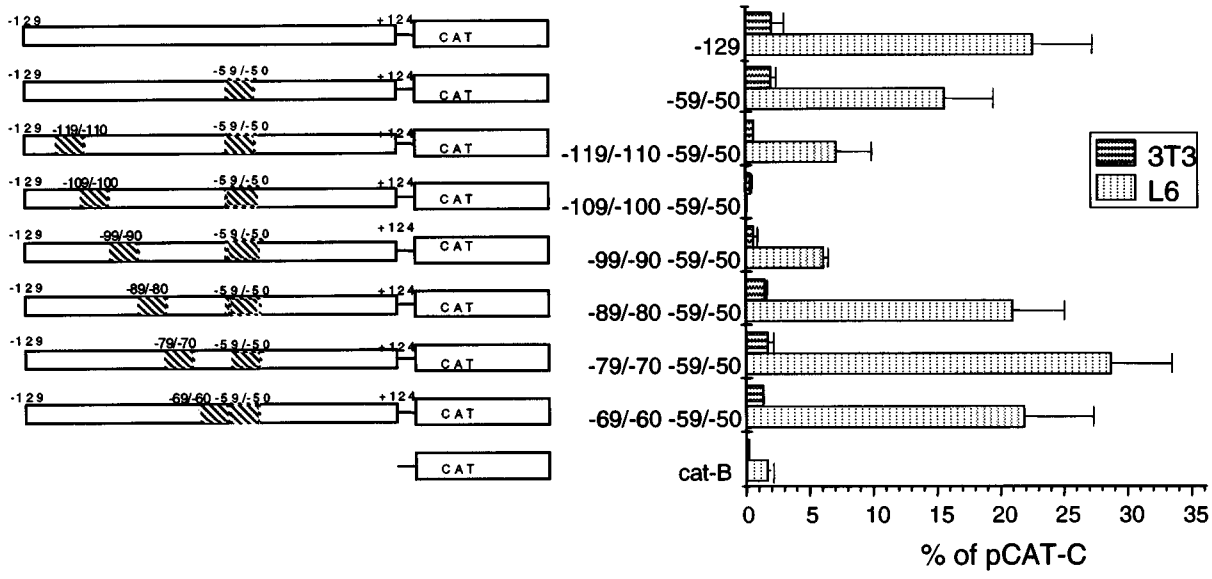
FIG. 3. Relative promoter activities of the scanning or deletion mutations in rSkM2 promoter. (A) Scanning mutations of –69/+1 segment in the context of the –129/+124 region. Sequences introduced by the 10-bp block mutations are shown below. (B) Alignment of the sequences in –129/–58 and –57/+1 show homology region between –113/–54 and –63/–7 segments. (C) The same scanning mutations shown in (A) are evaluated in the context of the –57/+124 segment. (D) Promoter activities from core promoter (–129/+124), proximal promoter (–57/+124), and distal promoter (–129/–50 or –129/–58) in L6 cells and NIH3T3 cells. Relative CAT expression calculated as described in the legend to Fig. 1. The promoter activities and error bars for each construct are averages from three separate transfection experiments.

segments containing GC-rich elements in distal (–113/–72) and proximal (–63/–26 or –63/–40) promoter segments were used in gel-shift assays with nuclear extracts from L6 myotubes and NIH3T3 cells (Fig. 5A). Both promoter elements produce multiple nuclear protein complexes, some of which appear to have comparable abundance and migration distances when incubated with either L6 myotubes or NIH3T3 nuclear extracts. Some nucleoprotein complexes formed with the distal and proximal promoter elements are clearly distinct from complexes generated with a Sp1 consensus probe (Fig. 5B), indicating the involvement of *trans*-factors other than Sp1. Supershift experiments with Sp1-specific antibodies con-


C
-57
+1
+124
CAT
-57/-50
-49/-40
-39/-30
-29/-20
-19/-10
-9/+1
WT
3T3
L6
cat-B
0
10
20
30
40
%of pCAT-C


D
-129
+124
CAT
-57
-129 -50/-10 +124
-129 -58/-10 +124
3T3
L6
Δ-49/-11
Δ-57/-11
cat-B
0
10
20
30
40
% of pCAT-C

-109/-100 is the essential *cis*-element in distal promoter



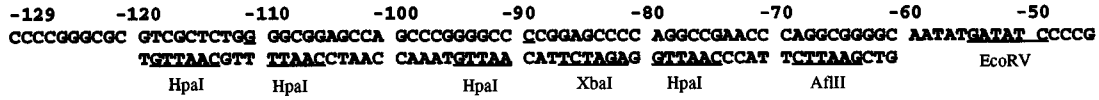


FIG. 4. Relative promoter activities of the scanning mutations in rSkM2 distal promoter. Scanning mutations of –119/–60 segment in the context of –129/+124 (containing the –59/–50 mutated segment that almost completely inactivates the proximal promoter, Fig. 3C). Sequences introduced by the 10-bp block mutations are shown below: the underlined segments represent introduced restriction sites for ease of screening. Promoter activities expressed relative to pCAT-C in L6 cells and NIH3T3 cells. Relative CAT expression calculated as described in the legend of Fig. 1.

firmed the presence of Sp1 in some complexes formed from both distal and proximal promoter elements (–113/–72 and –63/–26 or –63/–40, Fig. 5C). Our results suggest that the distal and proximal promoter elements bind similar sets of nuclear factors, and that the ubiquitous transcription factor, Sp1, is one of the components in some of the complexes.

Not all GC-rich motifs show the same behavior in supershift experiments with anti-Sp1 antibodies. The –28/+7 segment, which contains a Sp1 consensus site (Fig. 5A), forms multiple complexes, at least one of which appears to be muscle specific (Fig. 5B, band labeled "ms"). However, none of the complexes contains Sp1 because there are no supershifted bands in the presence of Sp1-specific antibody (Fig. 5C). This failure of a consensus Sp1 recognition site to recruit Sp1 protein indicates the critical role of the surrounding nucleotides in determining DNA binding to the Sp1 protein. Similarly, the GC-rich motifs contained in the –57/–44 and –28/+7 segments apparently do not support Sp1 binding and the +94/+113 probe, also containing a GC-rich motif, fails completely to bind muscle or nonmuscle cell nuclear factors (Fig. 5A, B).

Further evidence that the distal and proximal promoter elements bind similar sets of nuclear proteins was provided by gel-shift competition experiments. When labeled proximal promoter element (–63/–26) was incubated with nuclear extract from L6 myotubes, five complexes (A–E) were detected (Fig. 6A). All of these radiolabeled nucleoprotein complexes were disrupted by competition with either unlabeled proximal (–63/–26) or distal (–113/–72) promoter elements (Fig. 6A), although the distal promoter element appears to have somewhat lower affinity for the factors than does the proximal promoter element. Nuclear proteins in complexes C and E can also bind –28/+7 and –57/–44 DNAs because they can be competed by excess amounts of unlabeled –28/+7 and –57/–44 DNA segments. Complexes A, B, and D are

Sp1 related based on the ability of excess unlabeled Sp1 recognition site DNA to disrupt the bands. It may be that Sp1 and CCAC-like motifs comprise all the important *cis*-elements in the proximal promoter region (–63/–26) because a mixture of 25- and 50-fold excess of unlabeled Sp1 and –57/–44 DNAs, respectively, can disrupt virtually all of the complexes formed with the –63/–26 probe (Fig. 6A).

When the radiolabeled distal promoter element (–113/–72) is incubated with nuclear extracts from L6 myotubes, four complexes (labeled 1–4) are detected (Fig. 6B). All radiolabeled complexes can be disrupted by 50- to 100-fold excess of either unlabeled distal (–113/–72) or proximal (–63/–26) promoter elements: the proximal promoter element appears to have lower affinity for factor(s) in complex 4. Complex 4 appears to be formed not only with –113/–72 but also with –57/–44 (containing a CCAC-like motif) and –28/+7 DNA segments. The apparent order of affinity is –113/–72 ~ –28/+7 >> –63/–26 ~–57/–44. Consistent with the lower affinity of the proximal promoter element (–63/–26) described above, the –57/–44 segment is less potent in disrupting complex 4 than is the distal promoter DNA. Complexes 1–3 can be disrupted by unlabeled Sp1 probe, indicating that they contain Sp1 or related proteins.

Multiple Nuclear Factors Bind to the CCAC-Like Motif

Because the abundant binding of the ubiquitous transcription factor Sp1 was very likely to be obscuring our ability to examine the interaction of other factors with promoter *cis*-elements, we examined a proximal promoter element (–57/–44), containing the CCAC-like motif but devoid of Sp1 recognition sites in gel-shift assays (Fig. 5A). This region contains, we have already shown, a critical *cis*-element for rSkM2 promoter activity in L6 cells (Figs. 1 and 3C). Three complexes (a–c) were formed in gel-shift assay using the –57/–44 probe and nuclear extracts from L6 myotubes (Fig. 7A). One band (c) was formed with both muscle and nonmuscle (NIH3T3) nuclear extracts while two others (a and b) were formed only with nuclear extract from L6 myotubes. We therefore focused further study on complexes a and b, both of which were substantially disrupted in competition experiments by incubation with excess unlabeled –57/–44 DNA, but not with Sp1 DNA. None of the complexes was supershifted by Sp1-specific antibodies (Fig. 8), indicating that we have succeeded in designing the –57/–44 DNA segment such that Sp1 binding has been eliminated. Conversely, nuclear extracts from 3T3 and L6 myotubes appear to form complexes with the Sp1 consensus site equally well, and DNA containing the CCAC-like motif (–57/–44) fails to disrupt the complexes formed with the Sp1 recognition site DNA (Fig. 7A).

The Crucial –57/–44 Proximal Segment Recruits DNA Binding Proteins That Appear to Bind Also to the Distal Promoter Region

In gel-shift assays with the –57/–44 probe, complexes a and b can be substantially disrupted by competition with the distal promoter element (–113/–72), the proximal promoter element (–63/–26), and –28/+7 DNA (Fig. 7B). In the first and last cases a new band appears, migrating somewhat faster than complex b, that may represent a nucleoprotein complex formed with a *trans*-factor possessing lower affinity for these DNA sequences (a binding event that is only revealed when higher affinity proteins are sequestered by the excess unlabeled competitor DNA). Only complex b, formed with the –57/–44 probe, can be disrupted by our previously identified C-rich motif (–44/–29) (43), indicating that complex b may contain a common set of nuclear proteins that binds to both –57/–44 and –44/–29. None of the complexes can be competed by the downstream C-rich region (+94/+113) or the Sp1 consensus site, indicating that these complexes are specific for the CCAC-like motif and do not contain Sp1.

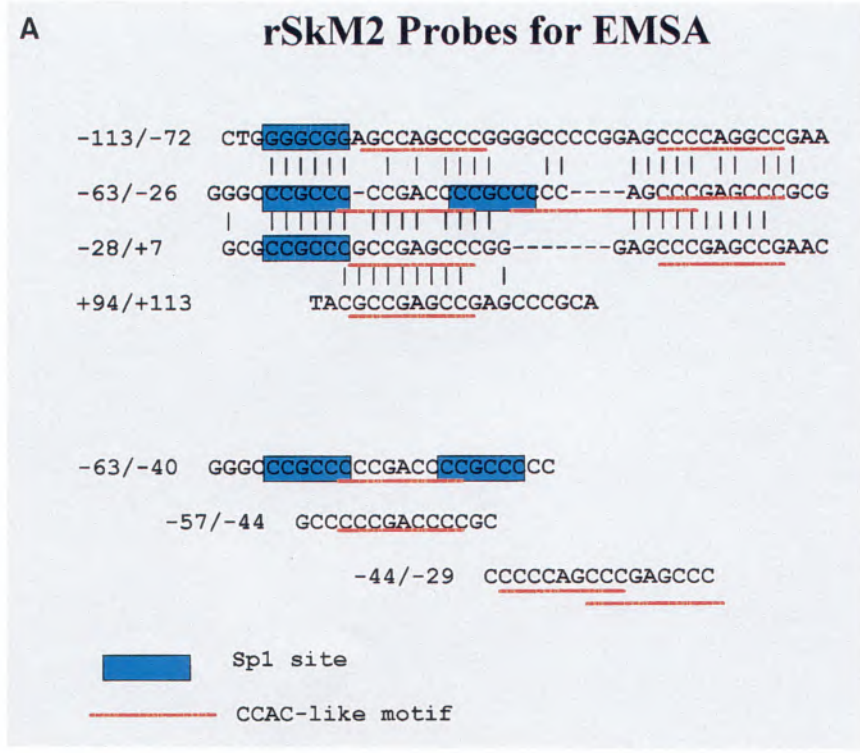
Several Nuclear Proteins Bind to the –57/–44 DNA Segment in UV Cross-Linking Experiments

UV cross-linking of L6 myotube nuclear proteins to the radiolabeled –57/–44 segment DNA revealed three proteins with approximate molecular weights of 22, 44, and 60 kDa (Fig. 7C) that were not observed either in the absence of UV irradiation or with irradiation in the absence of L6 nuclear extract.

To identify the nucleotides in the –57/–44 segment that are critical for complex formation, four probes with scanning trinucleotide transversion mutations were used as unlabeled competitor DNA to assess whether they could disrupt complex formation. The –57/–44 mut2 (GCCCAATACCCCGC) and, especially, mut3 (GCCCCCGCAACCGC) DNA probes failed to disrupt the formation of radiolabeled complexes a and b, indicating that nucleotides –53/–48 are critical for the binding of muscle nuclear factors to form those complexes (not shown).

Nucleoprotein Complexes Formed With –57/–44 Contain Factors That Bind to Other Muscle-Specific Gene Enhancers

The CCCGACCCC motif located at –54/–46 is very similar to the CCAC-box motif (CCCCACCCC)



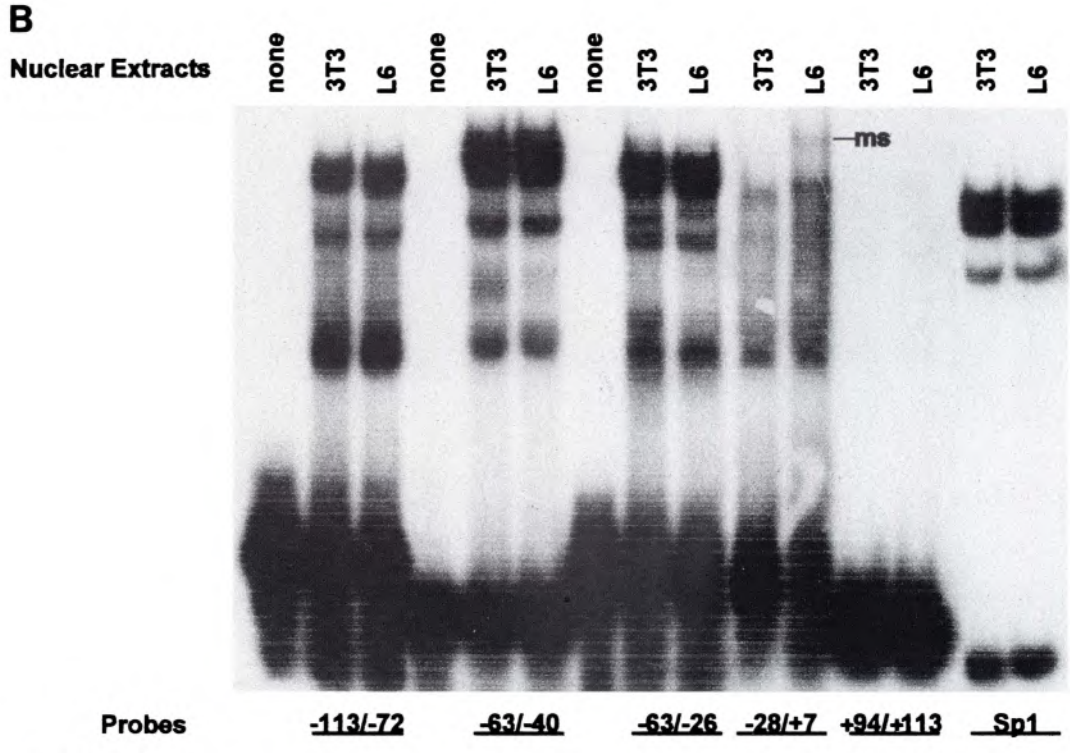


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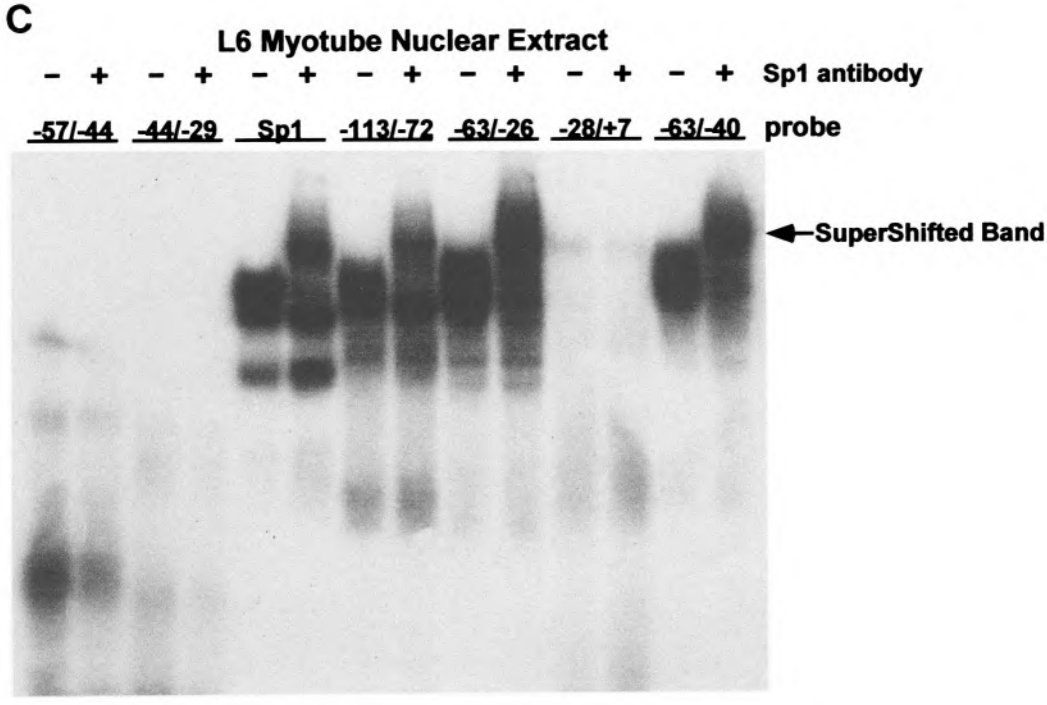
FIG. 5. Detection and identification of proteins bound to rSkM2 proximal and distal promoter elements. (A) Nucleotide sequences of distal promoter elements (−113/−72), proximal promoter elements (−63/−26, −63/−40, −28/+7), CCAC-like motifs (underlined) contained within the −57/−44, −44/−29, and downstream C-rich DNA segments (+94/+113). Boxed regions are Sp1 consensus DNA recognition sites. The numbering of the sequences is relative to the major transcription initiation site shown in Fig. 2. (B) Binding of L6 and NIH3T3 nuclear proteins to distal and proximal promoter elements and C-rich motifs identified by gel-shift assay experiments as described under Materials and Methods. The Sp1 consensus duplex oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′, Promega) was used as a nuclear extract activity control. "ms" signifies muscle-specific. (C) Supershift with Sp1-specific monoclonal antibody of some complexes formed with distal (−113/−72) and proximal promoter elements (−63/−26 and −63/−40). The binding reactions were incubated 30 min at 4°C with 2 μl Sp1-antibody before labeled probes were added.



contained in other muscle-specific gene enhancers (Table 1). The authentic myoglobin and muscle creatine kinase (MCK) CCAC-box motif DNAs disrupt nucleoprotein complexes formed with –57/–44 and L6 myotube nuclear extracts (Fig. 8), providing evidence that the –54/–46 segment of rSkM2 and other muscle-specific genes contain CCAC-like motifs that bind some of the same nuclear factors (although the MCK DNA seems to be somewhat less potent as a competitor). A mutation that abolishes the myoglobin CCAC-box nuclear protein binding and enhancer activities maps to the comparable site in the rSkM2 proximal promoter (see –57/–44 mutation 3 above). Thus, we conclude that the –57/–44 DNA segment in the rSkM2 promoter and the CCAC-box in the myoglobin enhancer bind one or more identical nuclear factors. In addition, the complex formed in L6 nuclear extract with the –57/–44 probe can be specifically supershifted by anti-MNF antibody (Fig. 8). In contrast, the antibodies raised to htβ (also a CCAC-box binding factor, cloned from T cells) (36,50), Sp1 (binds CCGCCC), and Rb (recognizes GCCACCC) (15) do not supershift the complexes (Fig. 8, lanes 7–9). This finding is evidence that MNF is a component in complexes formed between the L6 muscle cell nuclear factors and the rSkM2 CCAC-like motif contained in the –57/–44 segment. The fact that some bands are not supershifted suggests that unidentified nuclear factors other than MNF are involved in nucleoprotein formation with this DNA probe.

The –57/–44 Segment and MNF Alone Are Not Sufficient to Account for rSkM2 Expression in L6 Muscle Cells

To determine whether the critical –57/–44 segment in the proximal promoter, which binds both MNF and other muscle nuclear factors, is sufficient to direct muscle-specific gene expression, we introduced a tetramerized –57/–44 segment upstream of two ubiquitously expressing viral promoters [thymidine kinase (tk) or SV40] driving the CAT reporter gene. Multimerized promoter segments can amplify *cis*-element activities that otherwise may not be detected (45). The CCAC-like motif in rSkM2 decreases the tk promoter activity to 42% in L6 cells and to 20% in NIH3T3 cells, but does not significantly influence the activity of SV40 promoter in either cell line (not shown), suggesting that the –57/–44 segment is not sufficient by itself to positively influence these heterologous promoters. To determine the function of binding of MNF to –57/–44, we coexpressed MNF-α or its related protein, MNF-β, with rSkM2 promoter expression constructs (–129/+124 or –57/+124). Overexpressed MNF proteins failed to affect rSkM2 promoter activities in either

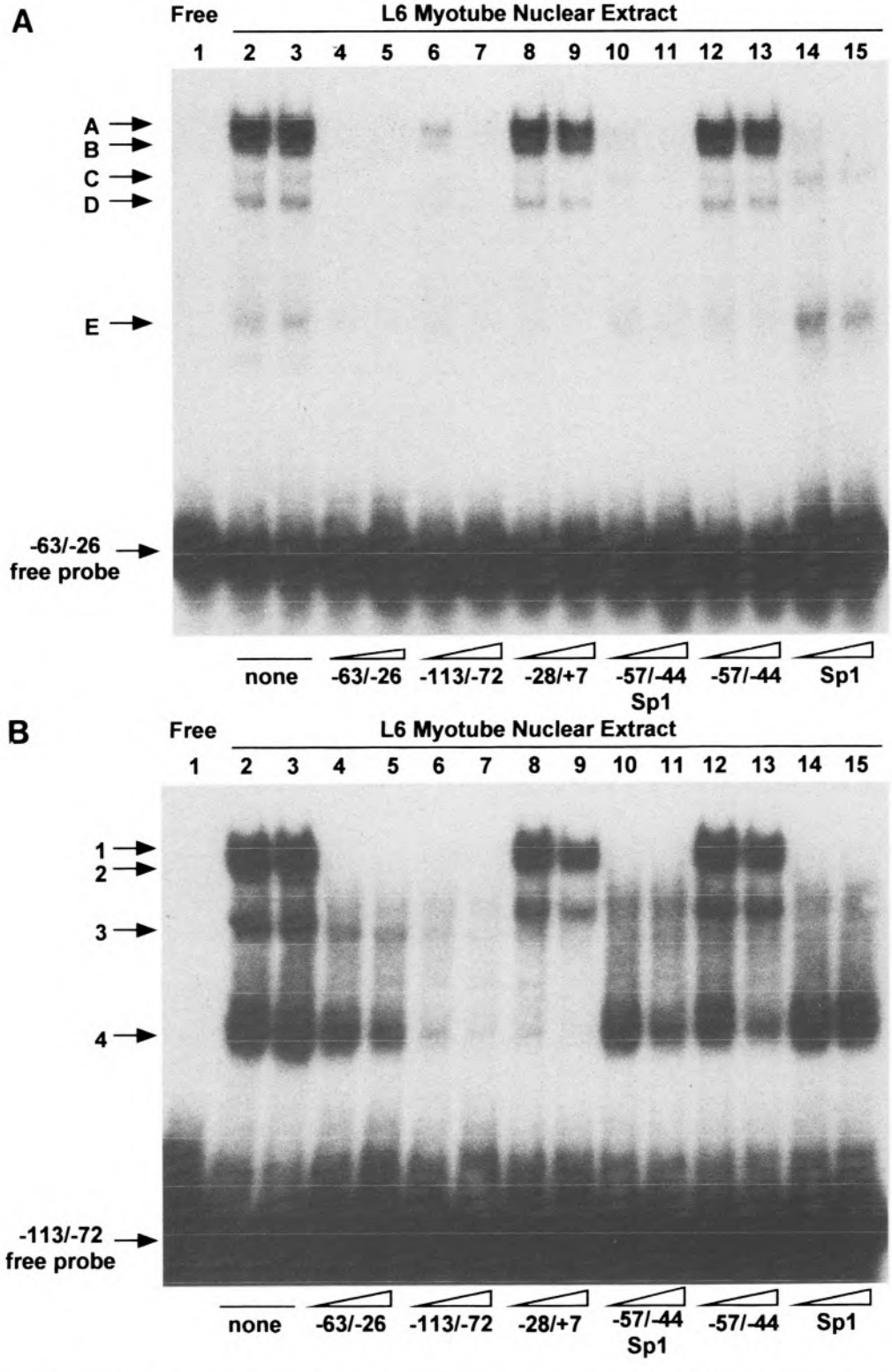


FIG. 6. Distal and proximal promoter elements bind the same or similar sets of nuclear proteins. (A) Labeled proximal promoter element detects complexes formed with nuclear extract from L6 myotubes. The competition was performed with 50- and 100-fold excess of unlabeled probe indicated at the bottom of the lanes. In the case of the mixture of −57/−44 and Sp1 oligonucleotides, a 25- and 50-fold excess of each unlabeled competitor were used, respectively. (B) A similar competition experiment using distal promoter element (−113/−72) as labeled probe.

L6 muscle cells or NIH3T3 cells (data not shown), consistent with a requirement for other muscle-specific factors to interact with rSkM2 promoter elements (perhaps via the CCAC-like motif).

Lack of Expression of rSkM2 in Nonmuscle Cells Is Not Due to Repression Mediated by the −29/−20 Segment

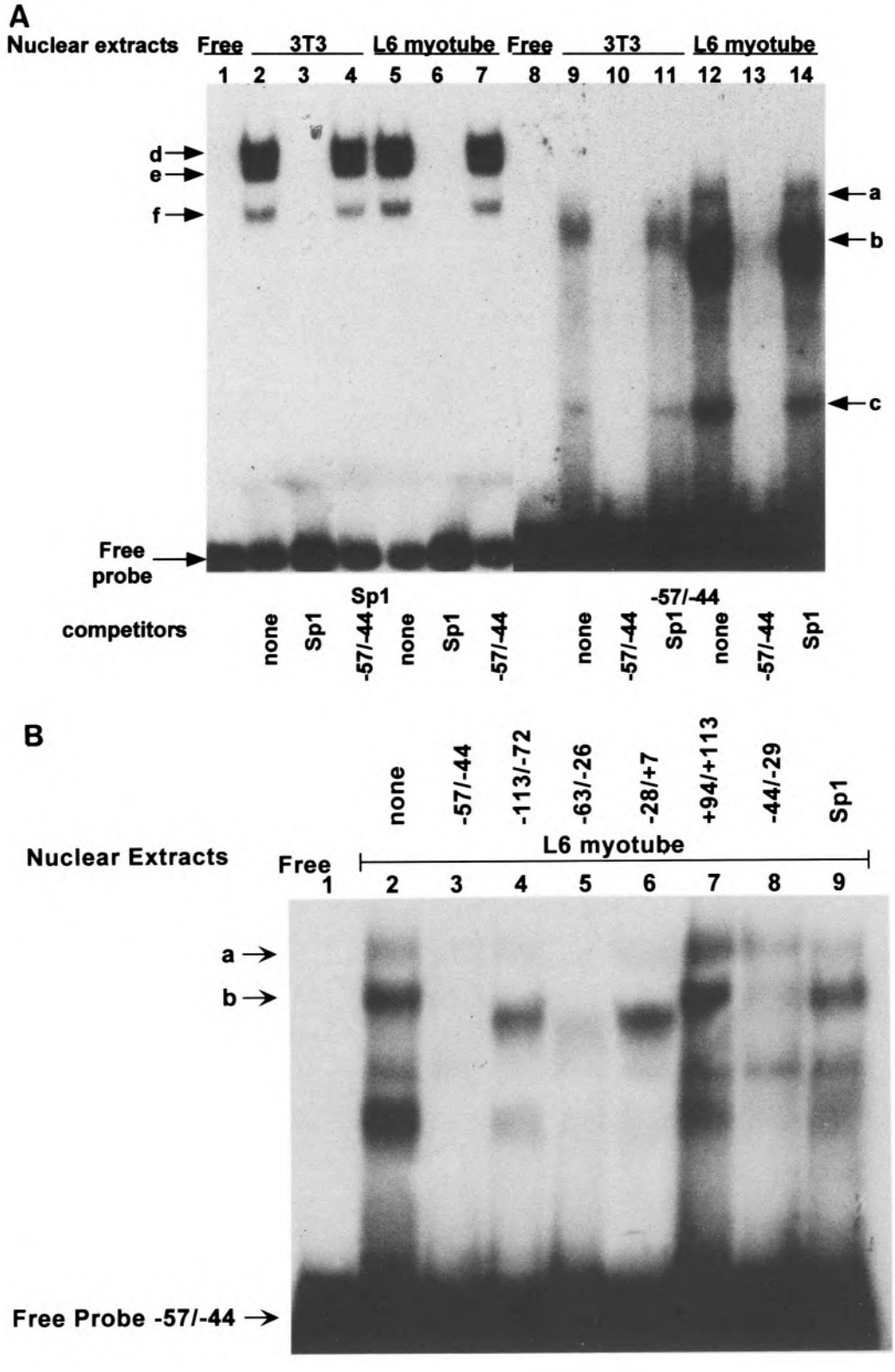
The three- to fivefold increase in rSkM2 promoter activity observed in NIH3T3 cells with mutation of the −29/−20 sequence (from CGCGCCGCCC to TAATATTAAA, Fig. 3A and C) suggested to us that this segment might play a negative role in nonmuscle cells (NIH3T3), and could contribute to rSkM2 L6 muscle-specific expression. However, mutation of the −29/−20 region to a different sequence (TTTTAAT TAA) in a luciferase reporter construct did not produce a significant increase of rSkM2 promoter activity in NIH3T3 cells (data not shown). This suggests that the increase in activity of the original −29/−20 mutation in the CAT reporter construct is not due to the removal of a negative element active in nonmuscle cells (NIH3T3), but may be an artifact of the sequence to which it was mutated. Further experiments are necessary to elucidate the mechanism of the upregulation caused by the original mutation.

DISCUSSION

The production of a cell membrane with specific electrical properties is a complex process resulting from the regulated expression of many ion channel genes, including voltage-gated sodium channels, such that the proper amount of each channel isoform is expressed at suitable times and in desirable amounts in the appropriate cell type. Most ion channel genes, including rSkM2, are expressed in a precise tissue-specific and developmentally defined manner but the underlying control mechanisms are just beginning to be elucidated. Both positive and negative transcriptional control have been shown to be responsible for tissue- and developmental-stage-specific expression of voltage-gated sodium channels (34,38,43). For brain sodium channels, expression is controlled by indiscriminate positive regulatory mechanisms in conjunction with negative regulatory processes that prevent transcription in inappropriate tissues (16, 28,31). Our group has shown that the expression of the TTX-sensitive skeletal muscle sodium channel (rSkM1) is controlled through interactions of multiple positive and negative regulatory elements in skeletal muscle (29). The mechanism is complex and involves positive *trans*-factors that bind to an E-box in the basal promoter region and recruit additional factor(s) to a second E-box in an upstream repressor region. In the present study, we have addressed aspects of the regulation of the second major skeletal muscle sodium channel gene—that encoding the TTX-resistant rSkM2 protein.

Sequence analysis of the −1961/+1 segment immediately upstream of the major transcription initiation site of the rSkM2 gene indicated that there are consensus sequences for several previously identified transcription factors. Within a larger (2.9 kb) fragment are positive (−2.9 kb to −1962 or 0.9 kb) and negative (−645/−506) upstream elements that, together with the −129/+1 core promoter segment, produce about the same level of transcriptional expression in L6 myotubes as does the −129/+1 segment by itself (43). Furthermore, the level of transcription increases for these reporter constructs as myoblasts are induced to form myotubes (43). The 0.9-kb positive element functions in a position- and orientation-independent fashion in the context of the −1961/+124 and −645/+124 promoter segments, but fails to increase transcription activity with a heterologous promoter (SV40) or the rSkM2 −129/+1 promoter devoid of the negative element. This finding suggests that within the 0.9-kb fragment there exists an antirepressor rather than an enhancer. At that stage of our studies we concluded that the expression of rSkM2 is controlled both by a tissue- and developmental-stage-specific core promoter and by interactions between the upstream positive and negative elements.

When we identified E-boxes (CANNTG) within the −1961/+1 upsteam segment, we anticipated that they might be involved in rSkM2 transcriptional control. The myogenic bHLH transcription factors (MyoD, Myf5, myogenin, and MRF4/Myf6) are known to activate skeletal muscle genes directly by binding to E-boxes in their control regions (13,20,37) or indirectly through the action of other myogenic transcription factors, such as MEF-2 (22,35,56). However, the −129/+1 segment of the rSkM2 promoter that controls muscle- and stage-specific expression contains neither E-boxes nor MEF-2 binding sites, a finding that has precedence in several other muscle genes such as cardiac troponin T (cTNT) and β-myosin heavy chain (33,47). Furthermore, even for genes that do contain E-boxes, the E-boxes may not be required for muscle expression, for example, myoglobin (3–5) and the δ-subunit of the nicotinic acetylcholine receptor genes (24,46). In addition, although the myoD family of transcription factors are expressed in skeletal muscle, they have not be detected in cardiac muscle where rSkM2 is prominently expressed, indicating that rSkM2 gene transcription can occur in the absence of the myoD family proteins (27,53). Given that the temporal and spatial pattern of rSkM2 ex-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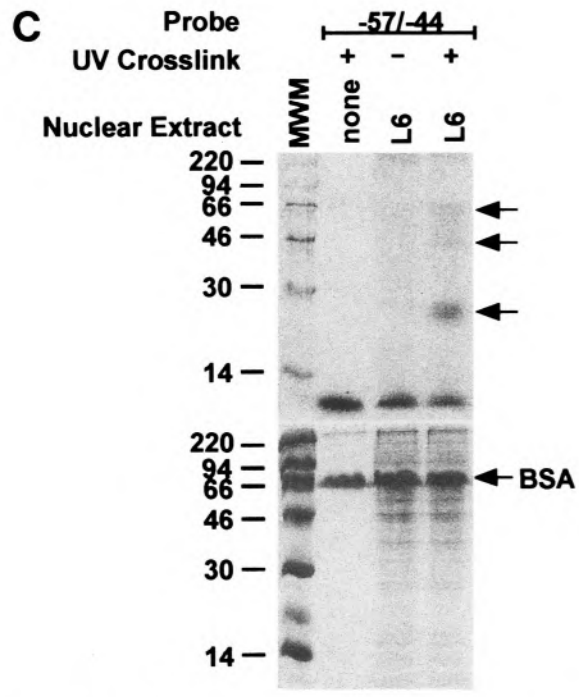

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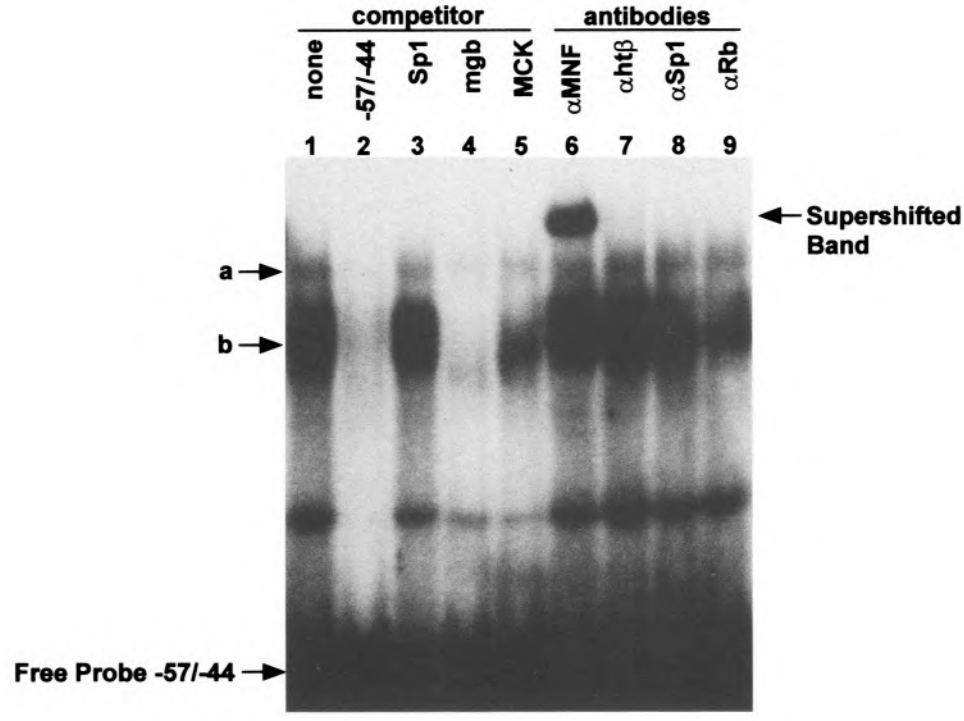
FIG. 7. Detection of nucleoprotein complexes formed with the −57/−44 probe. (A) Muscle-specific complexes a and b formed with nuclear extracts from L6 muscle cells (lane 12), but not with NIH3T3 nonmuscle cells (lane 9). The Sp1 consensus nucleotide is a control (lanes 1–7). (B) The competition of binding of nuclear proteins to the −57/−44 DNA segment in complexes a and b by distal promoter elements (−113/−72, lane 4), and proximal promoter elements [−57/−44 (lane 3), −63/−26 (lane 5), −28/+7 (lane 6)]. Complex b can be competed by −44/−29 (lane 8). Neither complex can be competed by Sp1 or the downstream C-rich motif (+94/+113) (lanes 9 and 7). (C) UV cross-linking of nuclear proteins in L6 muscle cells to the −57/−44 DNA segment. The same gel was stained with Coomasie blue (bottom panel).

pression is distinct from those of other skeletal muscle-specific proteins, it is reasonable that rSkM2 transcription is controlled by different factors than those involved in expression of sarcomeric proteins in skeletal muscle cells. In support of this possibility, MyoD family proteins failed to transactivate rSkM2 promoter constructs in our cotransfection experiments (data not shown).

Other transcription factor binding motifs that have been implicated in regulating skeletal muscle-specific transcription are: M-CAT (32,33), GArG (8), Sp1 (6,41), and CCAC-boxes (3). In this study we investigated the possibility that one or more of these *cis*-elements play a role in rSkM2 expression concentrating on the segment immediately adjacent to the major transcription initiation site. An analysis of the promoter region (−241/+1) immediately upstream of the transcription initiation sites of the rSkM2 gene reveals multiple consensus recognition sites for the general transcription factor Sp1 (CCGCCC), and for proteins that bind to CCAC-like motifs (CCCCAC-CCC), such as MNF (Fig. 2). An alignment of the upstream sequences from the TTX-resistant rat channel (rSkM2) and the corresponding human isoform (hH1) showed that the regions are highly homologous from −141 to −21 nucleotides, with an 11-bp insertion (containing one additional copy of the CCAC-like sequence and one more Sp1 recognition site) in the hH1 gene (Fig. 2). Such sequence conservation through evolution often reveals regions that are functionally important. Both human and rat promoter regions, therefore, contain a similar arrangement of Sp1 and CCAC-like motifs. Neither the role of these sites nor of other possible *cis*-elements in this region had been evaluated previously. We chose not to focus on Sp1 because it is a widely distributed transcription factor and unlikely by itself to be responsible for tissue- and developmental-stage-specific expression of the rSkM2 gene.

The presence of CCAC-like motifs in the −129/+1 segment seems very likely to be significant because they are present in enhancers of several other muscle genes, such as myoglobin (3,17), β-MHC (47), cardiac troponin C (40,42), myogenin (19), MCK (25, 44), and troponin slow and fast genes (39) (Table 1). MNF-α is a factor that binds to the CCAC-box and has been cloned from a cDNA expression library with the myoglobin CCAC-box as a probe (5). However, the function of MNF with respect to rSkM2 regulation was not obvious, because expression of MNF is upregulated in intact muscles subjected to chronic motor nerve stimulation (5), whereas mRNA levels of rSkM2 are very low in innervated muscle and increase dramatically following denervation (53). In addition, the interaction between MNF-α and the CCAC-box has been reported to be of much lower affinity than the binding of MNF-α to (A/T)(A/G)TAAA(C/T)A, a sequence later identified by DNA binding site selection (54). Furthermore, overexpression of MNF-α in L6 muscle cells and in NIH3T3 cells had no effect in terms of rSkM2 promoter-driven transcription in our hands. These observations seem to demand that muscle-specific factors in L6 muscle cells other than the MyoD and MNF families are involved in rSkM2 transcription.

The most striking finding from our present experiments is that there are dual tandem promoter elements located within the −129 to +1 segment. Promoter activity is lost in L6 myotubes with a 5′ deletion extending into the −57/−49 segment despite the fact that scanning mutations through the −69/+1 segment in the −129/+124 construct do not silence promoter activity. Most of the same scanning mutations in the shorter −57/+124 promoter construct, in contrast, substantially diminished transcription, espe-



myoglobin CCAC box: ACGCACAACCACCCCCACCCCCTGTGG
myoglobin CCAC mut3: ACCACCCCGGTACCTGTGGC
MCK CCAC box: TCACCCCCACCCCGGTGCA

FIG. 8. The CCAC-like motifs in –57/–44, myoglobin, and MCK genes bind common factors. Competition of complexes formed with –57/–44 in rSkM2 promoter by authentic CCAC-like motifs from myoglobin and MCK genes (lanes 1–5). Supershift of the complexes formed with the rSkM2 –57/–44 element with MNF-, htβ-, Sp1-, and Rb-specific antibodies. Supershifts were performed as described in the legend of Fig. 5C.

cially the –57/–44 subregion (see below). Evidently the presence of the distal segment (–129/–58) enabled rescue of promoter function when the proximal promoter contained mutations that were deleterious to expression. The most straightforward conclusion is that there are dual tandem promoter elements within the rSkM2 promoter. Direct experimental support for this conclusion is that, when tested separately, the distal (–129/–58 or –129/–50) and proximal (–57/+1) promoter segments each drive reporter gene expression in a muscle-specific manner: the strength of the proximal promoter region appears to be about fourfold greater than that of the distal element in the L6 cell assay. Consistent with similar roles in driving and regulating transcription, an alignment of the two regions reveals considerable homology. Finally, nuclear proteins from L6 myocyte nuclear extracts that bind to the proximal promoter elements (–63/–26 and –57/–44) also bind to the distal promoter (–113/–72) segment, providing further evidence that the segments are functionally similar.

Although there are reports of multiple promoters and alternative use of different exons in other genes including those that encode potassium channels (9–11,52), we are not aware of reports of dual tandem promoter elements immediately adjacent to each other. What are the possible benefits of such a gene organization? First, rSkM2 is a single copy gene and its product is important in producing the action potentials in heart and skeletal muscle. Malfunction of sodium channel proteins due to mutations in the human skeletal muscle type 1 and heart subtype 1 genes are responsible for diseases of excitability in humans (2,21). The dual promoter elements of rSkM2 may reduce the likelihood of loss of sodium channel expression, which would be catastrophic. Second, the production of an action potential in different tissues is a complex event, requiring the controlled expres-

sion of many different channel genes so that an appropriate amount of each channel is produced. The existence of dual promoter elements that bind similar but not identical sets of transcription factors may allow precise regulation of the expression of this gene in various tissues during development or denervation. In the *slowpoke* Ca^{2+}-activated K^+ channel gene (CAK), there are at least four promoters, each of which generates a transcript with a unique 5′ exon. Each promoter directs channel expression specifically in various tissues (9–11). It will be interesting to learn whether the rSkM2 gene uses the proximal and distal promoter elements differentially in controlling the expression of rSkM2, for example, in skeletal vs. cardiac muscle. Finally, multiple transcription initiation sites have been detected for rSkM2 within a 50-nucleotide region in skeletal and cardiac muscle tissues and cultured L6 cells (43). It is possible that the different promoter elements determine initiation from different sites in rSkM2 gene. The various mRNA species thus produced may have different stabilities, translation efficiencies, or localization within cells and, thus, determine the appropriate channel densities in different tissues or developmental stages (52). We will examine these possibilities in our future studies.

The –57/–44 segment, which appears to have the greatest importance in proximal promoter activity, does not, however, influence transcription from heterologous viral promoters. There are precedents for such failure of *cis*-elements to influence heterologous promoters even though they function in the context of their natural promoters (16,28). Consistent with the importance of the –57/–44 segment in transcriptional assays is the fact that this DNA segment binds nuclear proteins and forms two L6 cell-specific complexes (complexes a and b, Fig. 7A). We have been able to identify that MNF and Sp1 proteins are in the nucleoprotein complexes formed with *cis*-elements of the rSkM2 promoter. Because coexpression of MNF in transfection experiments failed to activate the rSkM2 promoter in either NIH3T3 or L6 cells, MNF by itself is not sufficient to induce SkM2 muscle-specific expression, implicating the involvement of other factors. Indeed, exposure of complexes to UV light cross-links 22-, 44-, and 60-kDa proteins to the –57/–44 fragment. Our present efforts are directed toward the identification of these factors.

In summary, we have found that a complicated regulatory system with multiple *trans*-factors and *cis*-elements, including two tandem promoter regions, controls the precise temporal and cell-specific expression of the rSkM2 sodium channel isoform.

ACKNOWLEDGMENTS

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