Natural and Synthetic DNA Elements with the CARG Motif Differ in Expression and Protein-Binding Properties

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DNA elements with the CC(A/T)₆GG, or CARG, motif occur in promoters that are under different regulatory controls. CARG elements from the skeletal actin, *c-fos*, and myogenin genes were tested for their abilities to confer tissue-specific expression on reporter genes when the individual elements were situated immediately upstream from a TATA element. The *c-fos* CARG element, also referred to as the serum response element (SRE), conferred basal, constitutive expression on the test promoter. The CARG motif from the myogenin gene was inactive. The skeletal actin CARG motif functioned as a muscle regulatory element (MRE) in that basal expression was detected only in muscle cultures. Muscle-specific expression from the 28-bp MRE and the 2.3-kb skeletal actin promoter was *trans* repressed by the Fos and Jun proteins. The expression and factor-binding properties of a series of synthetic CARG elements were analyzed. Muscle-specific expression was conferred by perfect 28-bp palindromes on the left and right halves of the skeletal actin MRE. Chimeric elements of the skeletal actin MRE and the *c-fos* SRE differed in their expression properties. Muscle-specific expression was observed when the left half of the MRE was fused to the right half of the SRE. Constitutive expression was conferred by a chimera with the right half of the MRE fused to the left half of the SRE and by chimeras which exchanged the central CC(A/T)₆GG sequences. At least three distinct proteins specifically bound to these CARG elements. The natural and synthetic CARG elements differed in their affinities for these proteins; however, muscle-specific expression could not be attributed to differences in the binding of a single protein. Furthermore, the MRE did not bind MyoD or the myogenin-E12 heterodimer, indicating that muscle-specific expression from this element does not involve a direct interaction with these helix-loop-helix proteins. These data demonstrate that the conserved CARG motifs form the core of a family of functionally different DNA regulatory elements that may contribute to the tissue-specific expression properties of their cognate promoters.

Functionally different promoters are often composed of DNA elements that are structurally similar. One example is the CC(A/T)₆GG, or CARG, motif that occurs in the genes for the interleukin receptor, myosin light-chain 1/3, dystrophin, all of the vertebrate actins, and the immediate-early genes which are rapidly induced by growth factors (8, 12, 18, 27, 33, 42, 50). Many of these sequences have been shown to mediate growth factor-inducible gene expression in quiescent, serum-depleted cells. This induction is characteristically rapid and transient. Because of this property, these sequences are generally referred to as serum response elements (SREs) (47). In addition, these sequences are required for the basal, constitutive expression of the *c-fos* and cytoskeletal (β and γ) actin genes (16, 29, 35). The CARG elements of the muscle-specific α -actin genes can function as SREs (44, 49, 51), and they are also essential for muscle-specific expression (5, 28, 54). The presence of CARG motifs in constitutive and muscle-specific genes raises questions about their roles in tissue-specific expression. This paradox can be explained by at least two hypotheses. First, despite the obvious structural similarities, CARG elements may be functionally different with regard to their basal, tissue-specific expression properties. Alternatively, these elements may be functionally identical, but they may respond differently depending on the context of the promoter in which they occur. In the latter hypothesis, the muscle-specific expression of the α -actin genes may not be regulated by CARG

elements, but these sequences would be required for basal transcription in a manner similar to the requirement for the TATA element.

Numerous studies of CARG motifs from different genes have resulted in conflicting reports regarding the functions and protein-binding properties of these elements. Some studies have analyzed individual CARG elements upstream from the TATA elements of truncated promoters to directly test the expression properties of these conserved sequences (29, 44, 48, 49, 51). Previously, we reported that CARG elements from the skeletal α -actin and *c-fos* genes were functionally different in a minimal test promoter (51). The skeletal actin CARG sequence behaved as a muscle-specific regulatory element (MRE) when it was inserted upstream from the *c-fos* TATA element, but the *c-fos* SRE gave rise to constitutive expression in this context. In contrast, other investigations found that the expression properties of a cardiac actin CARG element and the *c-fos* SRE were virtually indistinguishable (44, 49).

The tissue-specific expression and factor-binding properties of a series of natural and synthetic CARG elements were compared to evaluate the extent of functional differences between CARG elements. The CARG elements examined in this study were muscle-specific, constitutive, or inactive when they were inserted upstream from the *c-fos* TATA element. These elements differed in their affinities for binding to at least three distinct nuclear proteins that are ubiquitous. The proteins are (i) the serum response factor (SRF), also referred to as p67 and CBF; (ii) the muscle actin promoter factor (MAPF), also referred to as MAPF1, p62, and p62DBF; and (iii) the ternary complex factor (TCF), also referred to as p62 and p62TCF (2, 14, 36, 41, 47, 51). SRF

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and MAPF can bind directly to DNA, but TCF recognizes the *c-fos* SRE complex with SRF and forms a ternary complex with it. The interaction of TCF with CARG elements from other genes has not been reported. MAPF2, a factor found primarily in extracts from C2, L6, and Sol8 myogenic cell lines, has exactly the same specificity for binding as the MAPF activity analyzed in this study (53). The data presented here demonstrate that sequence differences within this family of CARG elements significantly influence the expression and factor-binding properties of these gene regulatory elements.

MATERIALS AND METHODS

Plasmid constructions. Chloramphenicol acetyltransferase (CAT) constructs were prepared by inserting 28-bp oligonucleotide duplexes into the unique *SaI* site that is directly upstream from the truncated mouse *c-fos* promoter, positions -56 to +109, and the CAT reporter gene (Δ 56CAT) (11, 51). All the inserted sequences were confirmed by dideoxy chain termination sequencing of the double-stranded plasmid using Sequenase (United States Biochemical). The palindrome sequences were determined by the chemical cleavage method. Oligonucleotides were prepared on an Applied Biosystems 380B DNA synthesizer with *SaI*-compatible ends for ligation. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and SEP-PAK C18 cartridges (Waters Associates).

The Δ 56GH plasmid constructs with the human growth hormone reporter gene were prepared by digesting the corresponding Δ 56CAT constructs with *HindIII* and *XbaI* to excise the entire heterologous promoter and subcloning these fragments into the *HindIII*-*XbaI*-digested p0GH plasmid (40). The resulting plasmids had test cassettes upstream from the truncated *c-fos* promoter and the growth hormone reporter gene. All plasmids were purified by Qiagen columns according to the directions of the manufacturer.

Cell culture and transfections. Primary cultures of pectoralis skeletal muscle, skin fibroblasts, and liver were prepared from 11-day-old chicken embryos (9). Cells were plated 18 h prior to the transfection procedure. Cells were grown in Primaria plates (Falcon) with minimal essential medium containing 10% horse serum and 1 or 4% chicken embryo extract. Cultures were put in fresh medium 1 h prior to transfection. The liver cultures were grown in 1% chicken embryo extract throughout the transfection process because high levels of chicken embryo extract were found to decrease the efficiency of transfection. Muscle cultures were initially plated with 4% chicken embryo extract, but upon transfection, the chicken embryo extract was reduced to 1% to enhance myocyte differentiation and minimize fibroblast proliferation. Fibroblasts were typically passaged prior to transfection experiments, and these cultures were continually grown with 4% chicken embryo extract. All transfections were performed by the calcium phosphate method (56). The medium-DNA-calcium phosphate mix remained on the muscle and liver cultures overnight, while the fibroblast cultures were exposed to the precipitated DNA for only 6 h. Precipitated DNA was removed by washing the plates with phosphate-buffered saline. Cells were transfected with 10 μ g of the test promoter DNA and 0.5 μ g of the internal control plasmid pXGH5, pSV2CAT, or CMV-luciferase, depending on the reporter gene used in the test plasmid. For example, test constructs with the CAT reporter gene were typically cotransfected with 0.5 μ g of pXGH5 and/or 0.5 μ g of the CMV-luciferase plasmid as internal controls. The plasmid

pXGH5 has the metallothionein promoter fused to the human growth hormone gene (40). The plasmid pSV2CAT has the CAT reporter under the control of the simian virus 40 (SV40) promoter and enhancer (13). The luciferase reporter is under control of the cytomegalovirus promoter and enhancer. For the CAT and luciferase assays, cells were harvested 36 to 48 h after transfection, and protein extracts were prepared as described by Gorman et al. (13).

Analysis of gene expression. Aliquots of media were collected from the Δ 56GH construct transfectants every 6 to 12 h after transfection, and the level of human growth hormone in the medium was determined by radioimmunoassay (Nichols Institute Diagnostics). CAT activities were determined by the method of Gorman et al. (13). Data from these assays were quantified by excising the acetylated and non-acetylated chloramphenicol spots from thin-layer chromatography plates and by counting in scintillation fluid. Alternatively, the thin-layer chromatography plates were directly exposed to phosphor screens, and the data were quantified with a Molecular Dynamics PhosphorImager. Luciferase assays were performed by mixing 20 μ l of protein extract with 100 μ l of luciferase assay reagent containing 20 mM Tricine, 1.07 mM (MgCO_3) $\cdot$ 5H $_2$ O, 2.67 mM MgSO $_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μ M coenzyme A, 470 μ M luciferin, and 530 μ M ATP. Luciferase activity was recorded by an LB 9501 Lumat luminometer (EG&G Berthold).

Probe preparation and DNA-binding assays. The probes used in the electrophoretic mobility shift assays were from synthesized oligonucleotide duplexes or were excised from plasmids. Probes from plasmids were prepared by digesting Δ 56CAT with *HindIII* and *PvuII* to excise the insert containing the test sequence. These fragments were either treated with calf intestinal alkaline phosphatase and end labeled with polynucleotide kinase and [γ - 32 P]ATP or end labeled with the Klenow fragment of DNA polymerase and α - 32 P-deoxynucleotides. Oligonucleotide duplexes were end labeled with polynucleotide kinase and [γ - 32 P]ATP and purified with Elutip-d columns (Schleicher & Schuell) according to the directions of the manufacturer. Typical electrophoretic mobility shift assays contained between 0.25 and 1 μ l of column fraction or cell extract and 0.05 ng of probe, 10 mM Tris (pH 7.5), 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 8% glycerol, and 0.1 to 1.0 μ g of poly(dI-dC) $\cdot$ poly(dI-dC) in a 10- μ l total volume. The H9 cell extracts, prepared by the method of Dignam et al. (6), were provided by M. Z. Gilman. Embryonic-chicken skeletal-muscle extracts and partially purified, heparin-Sepharose column fractions from these extracts were prepared as described previously (51). Changes in these binding reaction components are specified in the figure legends. Following a brief incubation at room temperature, binding mixes were loaded onto a 5% polyacrylamide gel, and electrophoresis was performed at 10 V cm $^{-1}$ in 22 mM Tris borate-0.5 mM EDTA buffer. Gels were dried and exposed to film for 10 to 12 h at -70°C with an intensifying screen.

In vitro transcription and translation. The RNA for E12 and myogenin were prepared from the E12R (30) and myogenin (3) plasmids. RNA was synthesized by using 2 μ g of linearized DNA and T3 RNA polymerase for 2 h at 37°C in the presence of RNasin, and the RNA was treated and translated as described by Murre et al. (30). The E12-myogenin heterodimer was formed by mixing the E12 and myogenin translation products at a ratio of 1:4, because a mixture with this ratio of components exhibited optimal binding to DNA. DNA-binding reactions with these materi-

FIG. 1. *c-fos* SRE, skeletal actin MRE, and myogenin CARG sequences and structure of the truncated *c-fos* promoter. The SRE is from the human *c-fos* gene (position -296 to -323). The MRE is from the chicken skeletal α -actin gene (position -73 to -100). The myogenin CARG element is from the myogenin promoter at approximately -300. These 28-bp CARG elements, with *Sal*I restriction enzyme-compatible ends, were inserted into a unique *Sal*I site situated at position -56 of the truncated *c-fos* promoter that was upstream from the CAT or human growth hormone reporter genes (referred to as Δ 56CAT or Δ 56GH, respectively). Other features of these test promoters include a TATA element and the reporter gene at position +109 of the *c-fos* promoter. CAT constructs also contain the SV40 polyadenylation signals immediately downstream of the reporter gene.

als were incubated at room temperature for 1.5 h, and reaction mixtures contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 1.0 μ g of poly(dI-dC) $\cdot$ poly(dI-dC).

RESULTS

Basal expression of CARG elements from *c-fos* skeletal actin, and myogenin promoters. Conflicting conclusions regarding the tissue-specific expression properties of CARG elements were reported in previous studies (44, 49, 51). To test for differences in tissue-specific expression, 28-bp sequences of CARG elements from the *c-fos*, skeletal actin, and myogenin promoters were inserted upstream from the *c-fos* TATA element and the CAT reporter gene (Fig. 1). Basal, noninduced expression from these constructs was compared in chicken embryo primary cultures by using a transient-expression assay. Primary cultures of myocytes, liver, and fibroblasts were used in this study because muscle-specific expression from the skeletal actin promoter is at least 10-fold higher than the levels observed in the C2 or L6 myocyte cell lines (52). Significant levels of CAT activity were conferred by the *c-fos* CARG element (SRE) in the transfected fibroblast and myocyte cultures (Fig. 2) and in primary liver cultures (see below). These data are consistent with reports that the SRE, which is upstream from the TATA box, can function as a constitutive promoter element in fibroblasts (29, 48). The skeletal actin CARG element activated expression in transfected muscle cultures, but the construct was inactive in fibroblast cultures (Fig. 2) and liver cultures (see below). These data confirm a previous report that the 28-bp

FIG. 2. Properties of the *c-fos* SRE, skeletal actin MRE, and myogenin (MyoG) CARG elements in transient-expression assays in primary cultures of embryonic-chicken skeletal muscle and passaged fibroblasts. Cultures were transfected with SRE/ Δ 56CAT, MRE/ Δ 56CAT, or myogenin-CARG/ Δ 56CAT constructs. In the left panel, a constant volume of extract was assayed for each cell type, and the ratio of CAT expression from the MRE relative to that from the SRE was 1.2 in muscle and 0.002 in fibroblasts when the CAT data were calibrated to the level of growth hormone expression from the cotransfected internal control plasmid. In the right panel, the quantity of extract used in the CAT assays was adjusted so that the level of the growth hormone internal control was constant. For the myogenin-CARG/ Δ 56 constructs, 1 $\times$ indicates a single element and 2 $\times$ indicates two elements. All elements were in their wild-type orientations.

skeletal actin fragment, which contains the CARG motif, can function as an MRE (51). The ratios of CAT activity from the MRE and SRE constructs could be directly compared after differences in transfection efficiency were adjusted for by the cotransfection of an internal control plasmid (see Materials and Methods). In primary cultures of skeletal muscle, the SRE/MRE ratios of reporter gene expression were 0.8, 1.0, 1.2, and 2.7 in four separate experiments. In primary cultures of liver and fibroblasts, little if any detectable expression above background was obtained from the MRE construct, while the SRE construct activated reporter gene expression to levels that were 10- to 100-fold higher. These data demonstrate a functional difference in the basal, tissue-specific expression properties of the *c-fos* SRE and the skeletal actin MRE.

A myogenin promoter element containing the CARG motif was analyzed. Myogenin, a myogenic determination factor, is under the control of a muscle-specific promoter (32a). During embryogenesis, cardiac actin expression either precedes or coincides with the expression of myogenin and MyoD in the myotomal cells of the first somites and in limb buds (39). This early cardiac actin expression may indicate that the promoters of MyoD, myogenin, and the muscle-specific actins respond to similar regulatory signals. Analysis of the mouse myogenin promoter revealed a putative CARG motif approximately 300 nucleotides upstream from the start of transcription. This sequence has a dyad of symmetry and matches the CARG consensus of CC(A/T)₆GG except for a single G $\cdot$ C base pair within the A/T region (Fig. 1). Despite this homology, the myogenin CARG element did not activate expression when it was cloned upstream from the *c-fos* TATA element (Fig. 2). No reporter gene expression was detected above background in transfected skeletal muscle cultures with constructs containing either one or two myo-

FIG. 3. Expression from the skeletal actin promoter and the MRE is *trans* repressed by Fos and Jun in skeletal muscle. Four micrograms of the test plasmid, MRE/ Δ 56CAT, SRE/ Δ 56CAT, or the 2.3-kb skeletal actin promoter upstream from CAT was transfected with 2 μ g of pSVFOS and 2 μ g of pSVJUN (+) or with 4 μ g of the control plasmid, pSV (-) (20).

genin CARG elements. The myogenin CARG constructs were also inactive when transfected into primary cultures of liver cells (not shown).

Skeletal actin MRE is *trans* repressed by Fos and Jun in muscle. The *c-fos* gene is negatively autoregulated by its protein product, Fos, and by the protein Jun, which forms heterodimers with Fos (20, 24). These proteins negatively control basal and serum-induced expression, and this effect is mediated by the SRE. Since basal expression from the skeletal actin MRE differs from that of the *c-fos* SRE, we tested whether the muscle-specific expression conferred by the MRE could also be *trans* repressed by Fos and Jun in muscle cultures. Fos- and Jun-expressing plasmids were cotransfected with the test plasmids. The combination of Fos and Jun decreased expression from the skeletal actin MRE, the *c-fos* SRE, and the 2.3-kb skeletal actin promoter in skeletal muscle cultures (Fig. 3). Individually, Fos and Jun were also capable of repressing expression (not shown). Fos and Jun decreased expression from the MRE by 3- to 26-fold in five experiments (average = 11-fold), and the 2.3-kb skeletal actin promoter was repressed from 2- to 13-fold in three experiments (average = 7-fold). Since the MRE is an essential element for skeletal actin expression (5, 51, 54), it appears that the MRE is a major target for Fos and Jun regulation within this gene (see Discussion).

MRE palindromes confer muscle-specific expression. The skeletal actin MRE has limited symmetry compared with the SREs of the *c-fos* and the nonmuscle actin genes. To test for functional differences in the left and right halves of the MRE, perfectly symmetrical 28-bp elements were constructed to either the right side (mre.RR) or the left side (mre.LL) of the element. These palindromes are shown in Fig. 4, where differences between the sequences of the natural and synthetic MREs are indicated by lowercase letters. In each palindrome, 9 of 14 nucleotides were changed on one half of the element. Only one of these substitutions occurred in the central CARG motif, while eight changes were made in the flanking sequences. A third palindrome, referred to as mre.RRmut, was constructed with symmetrical transversion mutations in the CARG element of the mre.RR palindrome.

Individual palindromes were inserted upstream from the TATA element of the truncated *c-fos* promoter, and transient CAT expression was compared with that of the wild-

Palindromes:

mre.LL $\overleftarrow{\text{GCCCCGACACCCAAATtTGGgtgtcGggc}}$
 $\overrightarrow{\text{CGGGCTGTGGGTTTAAaACCCacagCccg}}$

mre.RR $\overleftarrow{\text{cggCcgctcgCCAtATATGGCGACGGCCG}}$
 $\overrightarrow{\text{gccGgcagcGGTaTATACCGCTGCCGGC}}$

mre.RRmut $\overleftarrow{\text{cggCcgctcgCCAtATATGGCGACGGCCG}}$
 $\overrightarrow{\text{gccGgcagcGGTaTATACCGCTGCCGGC}}$

Chimeras:

mre[L]:sre[R] $\overleftarrow{\text{GCCCCGACACCCAAATTAGGACATCTGCG}}$
 $\overrightarrow{\text{CGGGCTGTGGGTTTAACTCTGTAGACGC}}$

sre[L]:mre[R] $\overleftarrow{\text{ACAGGATGTCCATATATGGCGACGGCCG}}$
 $\overrightarrow{\text{TGTCTACAGGTATATACCGCAGCCGGC}}$

mre[CARG]:sre[flank] $\overleftarrow{\text{ACAGGATGTCCAAATATGGACATCTGCG}}$
 $\overrightarrow{\text{TGTCTACAGGTTTATACCTGTAGACGC}}$

sre[CARG]:mre[flank] $\overleftarrow{\text{GCCCCGACCCCATATTAGGCGACGGCCG}}$
 $\overrightarrow{\text{CGGGCTGTGGGTATAATCCGCTGCCGGC}}$

FIG. 4. Sequences of synthetic CARG elements. Palindromes are symmetrical, inverted repeats in the right or left half of the chicken skeletal actin MRE. Chimeras fused portions of the skeletal actin MRE (shaded) with the *c-fos* SRE. All elements contain *Sal*I-compatible ends for subcloning.

type MRE and SRE sequences in different cell types (Fig. 5). In muscle cultures, the wild-type MRE was active in the forward and reversed orientations. The right-right and left-left palindromes and the natural MRE activated CAT reporter expression in muscle to similar extents, but the mutated right-right palindrome was completely inactive. In multiple experiments, the mre.RRmut insert reproducibly

FIG. 5. Tissue-specific expression properties of the symmetrical MRE palindromes. Embryonic-chicken skeletal-muscle or liver cultures were transfected with the test plasmid with no inserted sequences, the skeletal actin MRE, the *c-fos* SRE, or the synthetic, symmetrical palindromes in the Δ 56CAT test promoter as described in Materials and Methods. MRE 5' $\rightarrow$ 3' indicates that the element is in the wild-type orientation, while MRE 3' $\rightarrow$ 5' indicates the reverse orientation. The autoradiographs shown in the three panels were exposed to film for different lengths of time because the liver and skeletal-muscle cultures had different transfection efficiencies.

gave lower CAT levels than the sequence with no insert. The lower activity observed with the mutant sequence may result from the disruption of a weak, cryptic promoter sequence within the vector. Generally, *mre*.RR, *mre*.LL, and the wild-type MRE activated CAT expression in muscle cultures from 6- to 27-fold above that activated by the vector containing no insert. In transfected liver cultures, no activation of expression was detected with the MRE or with any of the MRE palindromes. However, reporter gene expression was elevated above background by the *c-fos* SRE insert in both culture types. When the expression from the MRE or MRE palindromes was calibrated relative to the level of expression from pSV2CAT (SV40 promoter-enhancer), there was a 60- to 105-fold-higher level of expression from these test promoters in muscle than in liver cultures (comparing the data from the central and right panels of Fig. 5). In contrast, the expression conferred by the SRE was only twofold higher in muscle than in the liver culture when it was calibrated relative to that of the SV40 promoter enhancer. These heterologous promoter constructions displayed the same tissue-specific expression properties when the growth hormone reporter gene was used in place of CAT or when fibroblast and muscle cultures were compared (not shown).

The factor-binding properties of the wild-type MRE and MRE palindromes were determined with an electrophoretic mobility shift assay using radiolabeled DNA fragments excised from the corresponding Δ 56CAT vectors (Fig. 6). MAPF and SRF are the predominant MRE-binding activities in extracts from embryonic-chicken skeletal muscle (51). The analysis of the *mre*.RR palindrome revealed a protein-binding pattern that was similar, if not identical, to that of the wild-type MRE. Competition experiments with nonlabeled probes confirmed that these complexes resulted from the binding of MAPF or SRF (not shown). In contrast, there was no detectable binding of MAPF to the left-left palindrome, and the interaction of the SRF with this probe could be detected only by using a partially purified fraction of SRF and longer exposure times for autoradiography (not shown). Finally, MAPF or SRF binding to the mutated right-right palindrome (*mre*.RRmut) was not detectable. From these data, it appears that high-affinity binding of MAPF or SRF is not required for muscle-specific expression but that a mutation that completely abolishes detectable SRF binding can be correlated with element inactivation.

MRE-SRE chimeras differ in expression and factor-binding properties. A series of chimeras between the skeletal actin MRE and the *c-fos* SRE were synthesized to further analyze the structural requirements for expression and nuclear factor binding by this family of DNA regulatory elements. The sequences of the 28-bp chimeric elements are shown in Fig. 4. Chimeras were constructed by fusing the left half of the MRE with the right half of the SRE (*mre*[L]:*sre*[R]) and vice versa (*sre*[L]:*mre*[R]). The CArG motifs of the SRE and the MRE were also exchanged to test the contributions of the flanking sequences in expression and factor binding. The synthetic element referred to as *sre*[CArG]:*mre*[flank] has the *c-fos* CC(A/T)₆GG motif flanked on either side by 9 bp from the corresponding sequences of the skeletal actin MRE. This chimera differs from the natural skeletal actin MRE at three positions in the central A/T-rich region. Similarly, the chimera *mre*[CArG]:*sre*[flank] differs from the *c-fos* SRE at three positions in the A/T-rich core.

The MRE-SRE chimeras were inserted upstream from the truncated *c-fos* promoter to test their abilities to confer tissue-specific expression. For these measurements, the human growth hormone reporter gene was used in place of

FIG. 6. DNA-binding properties of the MRE and MRE palindromes. Electrophoretic mobility shift assays were performed with a chicken myocyte extract and DNA probes excised from the test promoter constructs. DNA probes contained portions of the truncated *c-fos* promoter (positions -56 to -19), the pBS linker, and MRE, *mre*.RR, *mre*.LL, *mre*.RRmut, or no inserted sequences. The positions of the MAPF and SRF complexes with these DNA probes are indicated with arrows. The identities of the MAPF and SRF proteins were determined by their relative electrophoretic mobilities and by their susceptibility to competition by specific sequences (not shown). Nucleoprotein complexes that are distinct from SRF and MAPF result from protein binding to the truncated *c-fos* promoter because they are detected with the DNA fragment that contains no insert. The identities and specificities of the proteins in these other complexes are not known. F indicates positions of free DNA fragments with or without inserts.

the CAT gene, and the rate of growth hormone accumulation in the medium was determined by radioimmunoassay (Fig. 7). Identical results were observed in three separate experiments. The same results were also obtained with CAT reporter gene constructions (not shown), but since multiple growth hormone data points were obtained from each transfected culture, smaller differences in chimeric element expression could be detected. Of the four chimeras, only *mre*[L]:*sre*[R] exhibited muscle-specific expression which was similar to that of MRE (Fig. 7). The three other chimeras gave expression time courses that were the same as or usually within a factor of 2 between the liver and skeletal muscle cultures. These chimeras, *sre*[L]:*mre*[R], *sre*[CArG]:*mre*[flank], and *mre*[CArG]:*sre*[flank], could be considered "SRE-like" in that they conferred constitutive expression on the test promoter. We note that the constitutive expression of both *sre*[CArG]:*mre*[flank] and *mre*[CArG]:*sre*[flank] demonstrates that the muscle specificity from this family of elements cannot be attributed solely to sequence differences in the central CArG motif.

The factor-binding characteristics of the chimeric elements were analyzed to determine the structural requirements for protein binding. The relative affinities of the MAPF and SRF proteins were evaluated by quantitative competition experiments using a ³²P-MRE probe excised from the MRE/ Δ 56CAT construct and nonlabeled oligonu-

FIG. 7. Time course of human growth hormone expression from constructs containing natural and synthetic CARG elements in the $\Delta 56$ GH test promoter. Expression was analyzed in cultures of transfected muscle (open boxes) and of liver cells (closed boxes). To account for differences in transfection efficiency, the growth hormone data were adjusted to the level of luciferase expression from a cotransfected internal control plasmid (see Materials and Methods). Time indicates the period following the addition of fresh medium after the transfection procedure. Growth hormone (GH) levels are reported as nanograms per milliliter divided by the light units of luciferase activity produced by the cotransfected internal control plasmid.

cleotide duplexes of the natural and chimeric elements (Fig. 8A). All of the chimeras were bound by SRF, but this factor appeared to have a lower affinity for mre[L]:sre[R] probe. The binding of MAPF was more sensitive to sequence differences between the elements. MAPF had the highest affinity for the natural MRE and the sre[L]:mre[R] chimera. In contrast, the affinity of MAPF for the *c-fos* SRE was lower by a factor of 30 (51; Fig. 8A). The chimeras mre[CARG]:sre[flank] and sre[CARG]:mre[flank] had low affinities for MAPF that were similar to that of the natural *c-fos* SRE. No binding of MAPF to mre[L]:sre[R] could be detected.

The interaction of TCF with these elements was also analyzed. TCF does not bind to DNA directly but forms a

ternary complex with SRF and the *c-fos* SRE (41). In this study, TCF complexes were detected with SRE, sre[L]:mre[R], and mre[CARG]:sre[flank] by using direct binding analyses (Fig. 8B). TCF did not form a detectable complex with the wild-type MRE, mre[L]:sre[R], or sre[CARG]:mre[flank]. These data are consistent with methylation interference footprint data which show that the sequences critical for the TCF interaction occur immediately upstream of the CARG motif in the *c-fos* SRE (41). Finally, a previously unidentified complex with a mobility slightly faster than that of the SRF complex was detected with the mre[L]:sre[R] element. These data rule out the involvement of TCF in muscle-specific expression and further demonstrate the complexity of the protein-nucleic acid interactions that occur with the CARG regulatory elements.

Skeletal actin MRE confers muscle-specific expression but does not bind to MyoD or myogenin. Numerous muscle-specific genes are under the direct control of the MyoD family of helix-loop-helix proteins (3, 21, 55). These proteins initiate myogenesis and also bind to E-box consensus sequences in the regulatory regions of muscle-specific genes. The binding of MyoD and myogenin to the skeletal actin MRE was analyzed to test whether muscle-specific expression from this element involves the direct binding of these helix-loop-helix proteins. The bacterially produced glutathione-MyoD fusion protein did not bind to a radiolabeled probe of the MRE, but it was capable of forming a nucleoprotein complex with the E-box probe from the creatine kinase enhancer (Fig. 9). This recombinant MyoD protein also does not bind to a larger fragment of the skeletal actin promoter (21). Recent reports suggest that the myogenic determination factors occur as heterodimers with the E12 protein in vivo (31). To test whether a helix-loop-helix heterodimer will bind to the skeletal actin MRE, myogenin and E12 were translated in vitro from their RNAs by using a rabbit reticulocyte extract. The translated products formed complexes with the creatine kinase E-box probe, but no specific binding to the MRE probe was detected (Fig. 9). The MRE probe formed a complex only with a preexisting, nonspecific DNA-binding protein in the lysate. Specific complexes with the E-box probe were detected with E12 and with the combination of E12 and myogenin but not with myogenin alone. Evidence that high-affinity DNA binding by myogenin requires the formation of heterodimers with E12 has been presented elsewhere (3, 4). These data indicate that the muscle-specific expression conferred by the MRE does not require the direct binding of helix-loop-helix proteins and suggest that the skeletal actin gene is under transcriptional control different from that of other muscle-specific genes.

DISCUSSION

Functionally different DNA regulatory elements containing the CARG motif. The CC(A/T)₆GG sequence, or CARG motif, occurs in the promoters of numerous genes which are under different regulatory control. These genes include *c-fos*, *Egr-1*, *Egr-2*, myosin light-chain 1/3, dystrophin, the interleukin receptor, and the family of muscle-specific α -actins and the cytoskeletal β - and γ -actins. In a previous study, it was shown that the skeletal actin MRE and the *c-fos* SRE, which contain central CARG motifs, were functionally different when cloned upstream from a TATA element (51). The skeletal actin MRE activated basal expression only in muscle, but the SRE activated expression in both muscle and nonmuscle cultures. These data provided evidence that the CC(A/T)₆GG sequence is the core motif in a family of

FIG. 8. Comparison of SRF, MAPF, and TCF binding to the natural and chimeric CArG elements. (A) Competition for complex formation between nuclear proteins and the skeletal actin MRE using no competitor (lanes 0) or 1 ng (lanes a), 10 ng (lanes b), or 100 ng (lanes c) of the indicated oligonucleotide duplexes. Electrophoretic mobility shift assays were performed with approximately 1 μ g of protein from a heparin-Sepharose column fraction of embryonic-chicken skeletal muscle and approximately 50 pg of MRE probe excised from the MRE/ Δ 56CAT plasmid by digestion with *Hind*III and *Pvu*II. The MAPF and SRF nucleoprotein complexes are indicated. (B) Formation of SRF and TCF-SRF complexes with oligonucleotide duplex probes. Electrophoretic mobility shift assays were performed under conditions that were optimal for the detection of the ternary complex. Reactions contained 50 pg of oligonucleotide duplex probe, 0.3 μ g of poly(dI-dC) $\cdot$ poly(dI-dC), and approximately 10 μ g of H9 T-lymphocyte nuclear extract. An H9 extract was used for this experiment because it has a relatively high level of TCF activity.

functionally different DNA regulatory elements. Further support for this hypothesis is presented in this study, which compared the properties of a series of synthetic 28-bp elements containing central CArG motifs. The expression and factor-binding properties of these elements are summarized in Fig. 10.

The skeletal actin MRE has a limited dyad of symmetry compared with the *c-fos* SRE (Fig. 1). The significance of the limited palindrome in the 28-bp muscle actin element was investigated by testing the effects of inverting its orientation

and of introducing nucleotide substitutions that extend the inverted symmetry. Expression from the MRE in muscle cells was not orientation dependent (Fig. 6A; 51), suggesting a functional significance to the inverted repeat. Perfectly symmetrical 28-bp palindromes constructed to the right or left half of the MRE were made by altering 9 nucleotides in the MRE sequence. Both palindrome constructions activated expression in muscle but not nonmuscle cell cultures. The tissue specificity of expression from these palindromes was indistinguishable from that of the parental MRE. These data suggest that despite numerous sequence differences, inverted repeats of the left and right halves of the MRE contain equivalent information for muscle-specific expression. These results are surprising in view of the significant differences in the nuclear factor-binding properties of these elements (see below).

Analyses of chimeras between the *c-fos* SRE and the skeletal actin MRE revealed structural requirements for muscle-specific expression. A chimera constructed with the left half of the SRE and the right half of the MRE conferred constitutive expression on the test promoter. It should be noted that this constitutive chimera, sre[L]:mre[R], and the muscle-specific palindrome mre.RR have identical CArG motifs, with a sequence of CCATATATGG. When these two elements are compared, it can be concluded that differences in the flanking sequences can give rise to differences in tissue-specific expression. However, analyses of other chimeras revealed that the sequence of the central CArG motif can also be important in determining the tissue-specificity of expression. For example, the chimera mre[flank]:sre[CArG] confers constitutive expression, but it differs from the muscle-specific MRE by three transversion mutations that occur within the central A/T-rich region of the CArG motif. The importance of these central A/T rich sequences is also indicated by the lack of expression from the myogenin CArG motif (Fig. 2). The inactivity may result from the presence of a single G $\cdot$ C base pair within the central A/T-rich region, because assays of synthetic-element expression suggest that numerous sequence combinations can be tolerated as long as the central CC(A/T)₆GG configuration is intact.

FIG. 9. The skeletal actin MRE does not directly bind to MyoD or myogenin. Helix-loop-helix proteins were tested for their abilities to form complexes with the skeletal actin MRE or the 3' E box from the mouse creatine kinase enhancer (positions -1160 to -1139) in electrophoretic mobility shift assays. Binding of the bacterially produced MyoD-glutathione *S*-transferase fusion protein (Glu-MyoD), 50 pg of oligonucleotide duplex probe, and no poly(dI-dC) $\cdot$ poly(dI-dC). Binding of the *in vitro* translation products myogenin (MyoG), E12, and MyoG plus E12 is shown in the right panel. Also shown is binding from the rabbit reticulocyte lysate with no translation product. Binding was analyzed with 50 pg of MRE or E-box oligonucleotide duplex probes with 1 μ g of poly(dI-dC) $\cdot$ poly(dI-dC).

FIG. 10. Summary of binding and expression properties of the natural and synthetic CARG elements. Lines between the factor and the sequence indicate contacts at base pairs that were identified by methylation footprinting with the natural skeletal actin MRE and *c-fos* SRE sequences (11, 36, 41, 47, 51, 53). The core CC(A/T)₆GG motifs are shaded in both elements. + or low, detectable protein binding to the natural or synthetic elements; - or nd, complete absence of detectable binding; nd, not determined. For MAPF, low indicates that the apparent binding affinity was more than 10-fold lower than that for the skeletal actin MRE as determined by quantitative competition assays. For SRF, low indicates that the apparent binding affinity was more than 10-fold lower than that for the *c-fos* SRE. For the palindrome elements, relative binding affinities were assigned on the basis of large differences observed in the intensities of the protein-DNA complexes. In other studies, SRF has been referred to as p67 and CBF; MAPF has been referred to as MAPF1, p62, and p62DBF; and TCF has been referred to as p62 and p62TCF. MAPF2, a nuclear protein found primarily in C2, L6, and Sol8 myocyte extracts, has the same specificity for binding as the MAPF activity analyzed in this study (51). Multiple CARG box-binding activities have also been identified in *Xenopus* embryos (45). Judging by electrophoretic mobility and DNA-binding characteristics, the embryonic factor ECF1 may be related to MAPF2, while ECF3 may be related to MAPF1.

No functional differences between the *c-fos* SRE and the proximal cardiac actin CARG element were detected in a previous study (49). It was also reported elsewhere that the SRE and the cardiac actin CARG both function as muscle-specific DNA regulatory elements (49). It is not clear from these results how the two elements contribute to differences

in the transcriptional regulation of their cognate promoters. During myocyte differentiation, basal expression from the *c-fos*, β -actin, and γ -actin genes decreases, while α -actin and other muscle-specific genes are activated (7, 26, 34). Furthermore, elevated *c-fos* expression will inhibit myocyte differentiation, as do sustained increases in the level of serum growth factors. Thus the activation of an α -actin gene by a serum-responsive DNA regulatory element would be unexpected under these conditions. It is possible, however, that the cardiac actin CARG element functions as a constitutive SRE-like element and that it does not directly contribute to the muscle-specific regulation of the cardiac actin promoter. From this argument it follows that other regulatory sequences within the cardiac actin gene function as the predominant regulatory switch during myocyte differentiation. It may be important that MyoD and myogenin can *trans* activate the human and chicken cardiac actin genes, and helix-loop-helix protein-binding sites containing the E-box consensus sequence have been identified within these promoters (10, 38). A MyoD-like protein may also be required for cardiac actin expression in *Xenopus* embryos (46). In contrast, there is little or no *trans* activation of skeletal actin expression by myogenin (10, 37), and these proteins do not bind to the MRE or a larger fragment of the skeletal actin promoter (21; Fig. 9). On the basis of these data, it appears that the cardiac actin gene can be directly regulated by helix-loop-helix proteins, while the skeletal actin gene is regulated by a different mechanism.

***trans* repression by Fos and Jun.** Overexpression of Fos in myoblasts inhibits muscle differentiation and the activation of muscle-specific genes, including the MyoD gene (22). Recent experiments have also shown that Fos can *trans* repress the *c-fos* gene through the SRE (12, 20, 24). Because the skeletal actin MRE confers muscle-specific expression and is structurally similar to the *c-fos* SRE, it was of interest to test whether this 28-bp MRE and the 2.3-kb skeletal actin promoter were sensitive to *trans* repression by Fos and Jun. Fos and Jun, independently and in combination, were capable of inhibiting expression from the 28-bp MRE and from the larger skeletal actin promoter. Since point mutations within the MRE completely inactivate the skeletal actin promoter (51, 54), it appears that this sequence is a major site for Fos and Jun regulation within this gene.

Fos and Jun can also prevent MyoD and myogenin from *trans* activating the creatine kinase enhancer (22, 23). The site of repression maps to the E-box consensus sequence, the binding site for MyoD and myogenin. Since the repression does not map to an AP-1 site, the inhibition by Fos and Jun probably does not involve the direct binding of these proteins to the enhancer. Similarly, the MRE and SRE sequences examined here lack the AP-1 consensus sequence. Repression and activation by Fos are independent functions mediated by different domains within the protein. The domain of Fos involved in the *trans* repression of immediate-early-gene CARG elements is distinct from the basic amino acid-leucine zipper domain which is required for binding to AP-1 sites (12, 57). Since DNA binding is apparently not required, repression may be mediated by protein-protein interactions between Fos (and Jun) and the factors that directly bind to the DNA regulatory elements. The elucidation of these protein-protein interactions may ultimately provide a link between the pathways that control muscle-specific expression through CARG elements and E-box sequences.

Complex interactions with multiple nuclear factors. Given the numerous regulatory properties associated with CARG

DNA elements, it is not surprising that they interact with multiple nuclear proteins. At least three proteins specifically bind to duplex probes of the *c-fos* SRE and the skeletal actin MRE. These proteins are SRF, MAPF, and a protein that we refer to as TCF. Figure 10 shows the orientations of these factors on the wild-type SRE and the MRE sequences as determined by methylation interference footprinting. The SRF binds symmetrically over the central CArG motifs (11, 47, 51). MAPF produces an asymmetric footprint on both elements (36, 51, 53). The affinity of the *c-fos* SRE is lower than its affinity for the MRE by a factor of 30. TCF cannot bind directly to the DNA but will form a ternary complex with the SRF and the SRE (41). The methylation interference footprint of this ternary complex is similar to the combined individual footprints of MAPF and SRF on the SRE. Multiple CArG box-binding activities have also been identified in *Xenopus* embryos (45).

Previous studies with the wild-type and mutated *c-fos* SREs found a correlation between TCF binding and the induction of transcription by serum (41) and phorbol esters (14). However, subsequent investigations found that ternary-complex formation was not required for induction by either of these agents (14, 19). In our experiments, the binding of TCF or MAPF also could not be correlated with cell type-specific expression (Fig. 10), but the complete absence of SRF binding correlated with the elimination of expression from CArG elements. Thus, the roles of TCF and MAPF are not clear, and the molecular basis for the functional differences between CArG elements is not known at this time. However, these data clearly demonstrate that sequence differences between CArG elements can give rise to strikingly different patterns of factor binding in vitro, and this can partially explain discrepancies between studies that have analyzed CArG elements from different genes.

The interaction of multiple nuclear proteins with CArG elements suggests that the regulatory properties conferred by these sequences may be controlled by more complex protein-DNA interactions than are apparent by footprinting or electrophoretic mobility shift assays. The constitutive and muscle-specific CArG elements described here have many features in common with the factors involved in differential regulation of α - and α -specific genes in yeast cells. The yeast PRTF protein is similar in amino acid sequence to the SRF; PRTF-binding sites tend to have the CC(A/T)₆GG motif; and the sequences flanking the CC(A/T)₆GG motif are required for the binding of MAT α 1, which interacts with PRTF (1, 15, 17, 32). We note that PRTF changes conformation when it is bound to regulatory sites in α -specific genes but not when it is bound to regulatory sites in α -specific genes (43). It is postulated that cell type-specific expression in yeast cells is regulated by DNA sequence-dependent changes in PRTF conformation and by the interaction of PRTF with proteins that bind to adjacent sequences. The situation in higher eucaryotes could be similar in that functionally different CArG elements may give rise to different binding protein conformations. Presumably, conformation differences would influence the interaction of CArG-binding proteins with other, as-yet-unidentified transcription components which may be cell type specific. Recently, the conformation of SRF was reported to be altered by phosphorylation, suggesting that cell type-specific differences in factor modification may contribute to the functional differences between CArG elements (25).

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