CAR_G boxes in the human cardiac α -actin gene are core binding sites for positive trans-acting regulatory factors

(transcriptional regulation/*in vivo* competition/gel mobility shift/chloramphenicol acetyltransferase assay)

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ABSTRACT Positively acting, rate-limiting regulatory factors that influence tissue-specific expression of the human cardiac α -actin gene in a mouse muscle cell line are shown by *in vivo* competition and gel mobility-shift assays to bind to upstream regions of its promoter but to neither vector DNA nor a β -globin promoter. Although the two binding regions are distinctly separated, each corresponds to a cis region required for muscle-specific transcriptional stimulation, and each contains a core CC(A+T-rich)₆GG sequence (designated CAR_G box), which is found in the promoter regions of several muscle-associated genes. Each site has an apparently different binding affinity for trans-acting factors, which may explain the different transcriptional stimulation activities of the two cis regions. Therefore, we conclude that the two CAR_G box regions are responsible for muscle-specific transcriptional activity of the cardiac α -actin gene through a mechanism that involves their binding of a positive trans-acting factor in muscle cells.

The cis-acting sequences that confer tissue-specific or developmentally modulated expression on their attached genes include enhancers and regulatory upstream elements interacting with positive (or negative) trans-acting factors in differentiating cells (for reviews, see refs. 1 and 2). The 5' upstream sequence of the human cardiac α -actin gene contains two cis-acting regions that are required for tissue-specific expression in muscle cells: a distal regulatory region [position -443 to -395; the numbers refer to base pairs (bp) upstream from the transcriptional start site at +1] and a proximal regulatory region (-177 to -47) (3, 4). A detailed analysis of the proximal regulatory region (4) showed that the DNA segments that contain a CC(A+T-rich)₆GG DNA motif, referred to as a CAR_G box, play the major roles as cis sequences required for muscle-specific transcription. The CAR_G motif is conserved and present one or more times in the 5' flanking sequences of the cardiac and skeletal α -actin genes and several other muscle-specific genes in vertebrates (3, 5). Furthermore, the two cis sequences in the proximal regulatory region of the cardiac α -actin gene could replace each other as positive sites, although the ability of the second region to stimulate transcription appeared weaker than that of the first. Thus, it was proposed that the two regions that encompass the CAR_G boxes interact with the same class of positive transcriptional regulatory trans-acting factors (4).

In the experiments reported here, we used an *in vivo* competition assay (6) to measure the transcription from reporter genes in which the cardiac α -actin promoter had been linked to a bacterial chloramphenicol acetyltransferase (CAT) gene (7). When such constructs and competitor DNA segments were reintroduced into proliferating muscle cells, only certain 5' flanking DNA fragments were able to bind trans-acting factors and specifically effect transcription from

the CAT gene. Our data reveal that the two regions responsible for the transcriptional stimulation, both of which contain the CAR_G box as a core sequence, are the only binding sites of putative positive trans-acting factors that we are able to detect. By using a gel mobility-shift assay, we have been able to identify a specific binding activity in nuclear proteins extracted from muscle cells but not from nonmuscle cells. Furthermore, since the *in vivo* competition experiments demonstrate that the binding site containing the second CAR_G box may have a weaker factor-binding affinity than the first one, these differential affinities may explain the different transcriptional activities of the corresponding cis-acting regions.

MATERIALS AND METHODS

Plasmid Construction. Methods used to construct various plasmids have been described (4). CAT gene expression plasmids, pHCA177CAT (3), pHCA122CAT, and pID2-(-108/-57)CAT (4) carry a 245-bp fragment (position -177 to +68), a 190-bp fragment (-122 to +68), and a 195-bp fragment (-177 to -108 and -57 to +68) of the human cardiac α -actin gene promoter (Fig. 1), respectively, adjacent to the CAT gene (7). As competitor plasmids, an *Eco*RI-*Hind*III (-177 to +68) fragment from pHCA177CAT, *Eco*RI-*Bgl* II (-177 to -48, -98, or -136) fragments from 3' deletion plasmids of the cardiac α -actin promoter, and a *Bgl* II-*Hind*III (-47 to +68) fragment from a 5' deletion plasmid, respectively, replaced corresponding restriction fragments of pML21mp10 (4). A *Bgl* II-*Hae* III (-113 to -84) fragment from a 5' deletion plasmid and a *Hae* III-*Bgl* II (-139 to -116) fragment from a 3' deletion plasmid, respectively, were inserted between *Sma* I and *Bgl* II sites of pML21mp10. These competitor fragments are schematically summarized in Fig. 1. A *Dde* I fragment (-166 to +73) of a mouse β ^{major}-globin gene promoter including a CAAT box (around position -75) from plasmid pMH β SV-330 LS-88/-79, kindly provided by T. Maniatis (Harvard University) (8), was filled in by the Klenow fragment and cloned into a *Sma* I site of pUC18.

Cell Transfection and CAT Assay. Mouse C2 myogenic cells (9) were grown in Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) fetal calf serum. CAT gene-expressing reporter plasmid DNA (0.1 μ g), mixed with a total 10 μ g of competitor and/or carrier plasmid (pML21mp10) DNA per 100-mm dish, was cotransfected into C2 myoblasts by the calcium phosphate precipitation method as described (3, 4). The cells were maintained as myoblasts for 48 hr after transfection and then collected for the CAT assay. Half of the cell extract was incubated for 3 hr and analyzed by thin-layer chromatography (3, 7). Quantitation of CAT

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Abbreviation: CAT, bacterial chloramphenicol acetyltransferase.

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FIG. 1. Assessment of the effect of competition with promoter segments derived from the cardiac α -actin gene on the reporter gene expression. The DNA fragments indicated by open bars and heavy lines from the human cardiac α -actin promoter were cloned and used as reporter genes and competitors, respectively. Three nucleotide sequences conserved among the human, mouse, and chicken cardiac α -actin promoters (open boxes) are displayed above (3). The underlined sequences are the CC(A+T-rich)₆GG sequences and the TATA box. The table to the right shows the level of CAT activities in the *in vivo* competition assay using three reporter genes. Detailed experimental conditions of the *in vivo* competition and plasmid constructions are described in the text. CAT activity of each reporter gene cotransfected with pML21mp10 vector DNA was taken as 100% activity. ND, not determined.

assays was performed by scintillation counting of the appropriate areas of plates. Each CAT activity data point represents at least six independent transfection assays using at least two different plasmid preparations in order to overcome the variability inherent in transfections.

Gel Mobility-Shift Assay. Nuclear extracts were obtained from C2 myoblasts or mouse Ltk⁻ cells (10) and tested in a modified gel mobility-shift assay (11). Each binding mixture (25 μ l) contained 0.5 ng of a 5'-end-labeled [³²P]DNA fragment from the cardiac α -actin gene promoter, 8 μ g of nuclear protein, and 1 μ g of poly(dI-dC) (Pharmacia P-L Biochemicals) in 10 mM Tris-HCl, pH 7.5/50 mM NaCl/1 mM dithiothreitol/1 mM EDTA/10% (vol/vol) glycerol. The assays were incubated at room temperature for 30 min and electrophoresed through 4% polyacrylamide gels in 6.7 mM Tris-HCl, pH 7.5/3.3 mM sodium acetate/1 mM Na₂EDTA.

RESULTS

***In Vivo* Competition Seen in Myoblasts.** We have shown that transcriptional modulating trans-acting factors in C2 cells were saturated by 1 μ g of DNA of a cardiac α -actin-CAT gene expression plasmid (e.g., pHCA177CAT) per 100-mm dish and, thus, that they are rate limiting (4). Such trans-acting factors are expressed and accumulate in myoblasts of C2 cells (12). When the CAT expression plasmid (reporter gene) was cotransfected into myoblasts together with a 100-fold excess of plasmid DNA containing the cardiac α -actin promoter segments (competitor DNA), some of these competitor constructs repressed expression of the reporter gene (4). As long as C2 cells were maintained at the myoblast stage, pHCA(-177/+68) carrying the same promoter fragment as the reporter gene pHCA177CAT efficiently blocked CAT activity as described (Fig. 2, lanes 1 and 2; ref. 4). In contrast, after the transfected cells were induced to differentiate into multinucleate myotubes, the competition effect was greatly reduced (Fig. 2, lanes 3 and 4). Thus, *in vivo* competition experiments with the cardiac α -actin gene can be performed well only in myoblasts. The small amount of CAT activity remaining after competition (lane 1) probably corresponds to expression from a promoter that does not have the proximal regulatory region [e.g., pHCA47CAT having only the basal promoter has 5% of the CAT activity of

pHCA177CAT (4); see Fig. 1]; thus, the competition by pHCA(-177/+68) appears to come from the binding of positive transcriptional factors that mediate muscle-specific expression through interactions with the upstream regulatory region.

Internal Fragments of the Proximal Regulatory Region Show Complete Competitive Activity. We searched for DNA segments that bind positive transcription factors in C2 cells by using pHCA177CAT as reporter gene and several 5' flanking segments as competitors as shown in Fig. 1. First, we investigated the effects of the known basal promoter sequences such as the TATA box, but the DNA segment between -47 and +68 in pHCA(-47/+68) had little effect in

FIG. 2. Transcriptional activity of the cardiac α -actin gene in mouse C2 myoblasts and myotubes. pHCA177CAT (0.1 μ g) and 10 μ g of pHCA(-177/+68) (lanes 1 and 3) or pML21mp10 (lanes 2 and 4) plasmid DNA were transfected in C2 myoblasts (3, 4). After 20 hr the medium was changed to normal growth medium, and the cells were allowed to proliferate as myoblasts for an additional 40 hr (lanes 1 and 2). Alternatively, the cells were switched to nutrient-poor medium that included 2% horse serum to induce differentiation into multinucleate myotubes (13) and then were incubated for 40 hr (lanes 3 and 4). The CAT assays were performed as described. Arrows and the arrowhead respectively indicate acetylated and nonacetylated forms of chloramphenicol.

competition. The failure of pHCA(−47/+68) to compete was not surprising, despite the fact that specific proteins that bind to sequences around the TATA box are known (14), since these DNA sequences have not shown significant *in vivo* competition activities in several expression systems (6, 15, 16). On the other hand, the competitive activity of pHCA(−177/−48), which carries the proximal regulatory region defined between −177 and −47, was identical with the competitive activity of pHCA(−177/+68). We have demonstrated previously that deleting sequences between −92 and −58 had no effect on cardiac α -actin gene transcription (4). Accordingly, we tested the ability of sequences upstream from this region to compete against pHCA177CAT. The corresponding plasmid, pHCA(−177/−98), competed strongly—almost as well as pHCA(−177/+68). None of these plasmids competed for the expression of the simian virus 40 viral promoter in the pSV2CAT (7) reporter gene construct (see ref. 4). Therefore, rate-limiting trans-acting factors in C2 cells, which may be promoter specific for the cardiac α -actin gene, appear to interact with the region between −177 and −98.

Two Regions Including the CARG Box Have Competitive Activity. Several lines of evidence (4) have shown that the two segments of the proximal regulatory region containing a CARG sequence—i.e., from −163 to −136 and from −119 to −94—are essential cis sequences required for tissue-specific transcriptional stimulation in the CAT assay. We examined whether these cis regions also show competition activity *in vivo*. The DNA carrying most of the first essential cis sequence from −113 to −84, pHCA(−113/−84), retained full competitive activity (Fig. 1), whereas the DNA segment between −139 and −116, pHCA(−139/−116), which does not seem to function in the regulation of transcription, had no effect in the competition assay. In addition, DNA carrying most of the second essential cis sequence from −177 to −136, pHCA(−177/−136), also competed against expression of the reporter gene. These results show that the two regions that lie at −177 to −136 and −113 to −84 can bind rate-limiting trans-acting regulatory factors in C2 cells. In addition, since each of these DNA segments can work as an independent site, they probably interact *in vivo* with separate trans-acting factor molecules.

The Same Factors Recognize Both Sites. Since the two cis-acting regions in the proximal regulatory domain can replace each other [e.g., pHCA122CAT and pID2(−108/−57)CAT(4)], the two regions seem to be similar functional sites required for tissue-specific transcriptional stimulation. To test the hypothesis that both regions interact with the same class of positive trans-acting factors, we examined the ability of the region around the second CARG box to competitively block the CAT activity of a reporter gene carrying only the first essential cis sequences. pHCA122CAT, which has the downstream region from −122 (Fig. 1), was tested in the presence of the same set of competitors. Since the promoter segment in pHCA(−113/−84) was fully competitive, this segment appears to be both the site binding the rate-limiting trans-acting factors in C2 cells as well as the dominant transcription-modulating segment of pHCA122CAT. Although this reporter gene construct does not carry the second CARG box region, DNA carrying this segment, pHCA(−177/−136), competes with this reporter gene. This result suggests that the trans-acting factors that can interact with the second site also function with the site present in pHCA122CAT. Conversely, both pHCA(−113/−84) and pHCA(−177/−136) competed with a reporter gene carrying only a sequence around the second CARG box as a modulating segment, pID2(−108/−57)CAT. Therefore, we conclude that the two sites around each CARG box bind the same transcription-stimulating factor or set of factors.

Two Sites Appear to Have Different Affinities for Factors. The experiments described above suggest that the two

regions interact with the same trans-acting factors and stimulate transcription in a tissue-specific manner. However, our previous results showed that pHCA122CAT and pID2(−108/−57)CAT had 58% and 31% of the transcriptional activity of pHCA177CAT, respectively (4); thus, the transcriptional activity of the second cis-acting region was consistently weaker than that of the first one. To investigate whether this difference in promoter strengths reflects different affinities for trans-acting factors, we compared the relative effect of different amounts of each of three competitors—pHCA(−177/−48), pHCA(−113/−84), and pHCA(−177/−136)—on CAT expression of pHCA177CAT. The reporter plasmid (0.1 μ g; 26 nmol) was cotransfected with competitor DNA in the range of 0.1 μ g (0.06 pmol) to 10 μ g (6.2 pmol). Carrier vector DNA (pML21mp10) was added to make up a final DNA concentration of 10 μ g per 100-mm dish. Cotransfections with these competitors progressively reduced the activities of the reporter gene, but their titration curves differed (Fig. 3). pHCA(−177/−48) had the strongest competitive activity at 3 μ g of DNA. This may be explained by the presence in pHCA(−177/−48) of both regions that interact with the trans-acting factors, whereas the other two competitor fragments only contain one of the two regions. The competitive activity of the second region in pHCA(−177/−136) was weaker than that of the first one in pHCA(−113/−84). Therefore, the binding affinity of trans-acting factors to the first site appears to be stronger than to the second site at the midpoint of this titration curve, and this difference in affinities may be responsible for the different transcriptional activities of these regions in C2 cells.

The CARG Box Is not the CAAT Box. Sequences that correspond to the first CARG sequence have been designated previously as a “CAAT” box (5, 17, 18), which is a constitutive upstream element in a number of eukaryotic promoters, although, as discussed previously (3), these sequences match poorly with the consensus CAAT sequence for CAAT-binding proteins (19). To test whether the positive trans-acting factors in C2 cells that recognize the two CARG regions can also recognize the true CAAT box, we used the promoter region of the mouse β major-globin gene (−166 to +73), which includes a well-characterized CAAT box (8), as a competitor. Since this plasmid did not have major competitive activity against the

FIG. 3. The effect of concentration of competitor DNA on reporter gene expression. For the competition assay with pHCA(−177/−48) (●), pHCA(−113/−84) (○), and pHCA(−177/−136) (◐), 0.1 μ g (26 nmol) of pHCA177CAT was cotransfected with a total of 10 μ g of competitor and/or vector pML21mp10 DNA. The percentage of activity relative to transfection with vector DNA was plotted against the amount of the three competitor plasmids. The numbers in parentheses show the picomoles of competitor DNA.

reporter genes (Fig. 1), we conclude that putative positive trans-acting factors for the CAAT box do not interact with a fragment around a typical CAAT box; thus, the function of the CAAT box must differ from that of the CAAT box.

Gel Mobility-Shift Assay Reveals Tissue-Specific Binding Factors. Nuclear extracts from C2 myoblasts were incubated with a 245-bp 5'-end-labeled DNA fragment of the cardiac α -actin promoter (−177 to +68). Two distinct bands of retarded mobility were seen (Fig. 4A), even in the presence of excess nonspecific competitor poly(dI-dC) at a concentration of 200 ng/ μ l (Fig. 4A, lane 10). Since this represented a 10,000-fold excess of poly(dI-dC) relative to the promoter fragment, the two bands seem to represent the interaction of sequence-specific binding factors with the cardiac α -actin gene promoter. Similar results were obtained with sonicated *Escherichia coli* DNA as the nonspecific competitor, but the two bands were slightly broader (data not shown). These bands were observed when nuclear extract from C2 myotubes was incubated with the same fragment (data not shown), while no bands were seen with nuclear extracts from mouse Ltk[−] cells (Fig. 4C) in which transfected cardiac α -actin CAT plasmids are not actively expressed (3). These findings are in agreement with our observation that transcriptional regulatory factors for the cardiac α -actin gene are expressed and accumulate in both C2 myoblasts and myotubes but not in fibroblast cells (12).

To determine whether the binding of nuclear proteins involved interaction with the upstream segment of the cardiac α -actin promoter, we repeated the experiment shown in Fig. 4A, but in the presence of increasing amounts of the unlabeled 245-bp promoter fragment (Fig. 4B, lane 2) or a 130-bp upstream fragment (−177 to −48) corresponding to the proximal regulatory region (lanes 3 and 4). Both of these DNA fragments specifically compete for the myoblast nuclear factors that bind to the cardiac α -actin gene promoter. Thus, the sequence-specific factors bind to the proximal regulatory region that is also required for tissue-specific transcription and is sufficient for *in vivo* competition.

DISCUSSION

Tissue-specific transcriptional modulation mechanisms involving the interactions of cis-acting sequences and positive or negative trans-acting factors in differentiating cells must be

distinguished from the activation events that allow a previously unresponsive gene to respond to the presence of such trans-acting factors. For example, transfected copies of the human cardiac α -actin gene promoter are expressed in a tissue-specific manner and at high levels in proliferating mouse C2 myoblasts, but the endogenous mouse cardiac α -actin gene is unresponsive to the tissue-specific factors before differentiation (3, 12). Thus, the transcription-modulating trans-acting factors cannot solely be responsible for activation of the cardiac α -actin gene. However, these trans-acting factors are expressed and accumulate in C2 myoblasts, which we used as the recipient cells in the *in vivo* competition assays. This *in vivo* competition worked well as long as C2 cells were in the myoblast stage, but complete competition could no longer be demonstrated clearly after differentiation into multinucleate myotubes. The loss of competition may be explained by the acquisition of excess trans-acting factors supplied to some of the transfected cells either by stimulation of their production or, more likely, by an influx from neighboring cells during myotube formation that had not taken up transfected DNA. This latter explanation is likely because (i) the number of cells transfected by the calcium phosphate precipitation method is low ($\leq 10^{-4}$) (our unpublished data), and (ii) the results of heterokaryon formation experiments show the ability of muscle-specific factors to migrate among nuclei in myotubes (20). Thus, the moderate degree of competition activity seen in myotubes is the sum of the activities expressed by residual transfected unfused myoblasts, transfected myoblasts that fuse rarely with each other, and, most commonly, transfected myoblasts that fuse with variable numbers of untransfected cells.

Sequences derived from the proximal regulatory region (−177 to −47) of the human cardiac α -actin gene repressed expression of a cardiac α -actin-CAT reporter gene to the level of a basal promoter in the *in vivo* competition assay. The simplest explanation for this observation is that such sequences bind positive regulatory factors. Indeed, the gel mobility-shift assay showed that some proteins with the expected characteristics bind to this upstream sequence: the proteins are present in the nuclear extracts of C2 cells but not of Ltk[−] cells, and the same DNA segments that compete in the *in vivo* competition assays compete for their binding. Furthermore, the activity of such factors on the cardiac α -actin gene promoter was not influenced by competitor

FIG. 4. Binding of nuclear proteins to the cardiac α -actin gene promoter and competition analysis. (A) Electrophoretic mobility-shift assay of the 5'-end-labeled 245-bp promoter fragment (−177 to +68) with nuclear extract from C2 myoblasts in the presence of various amounts of poly(dI-dC). Lanes: 1, free DNA fragment; 2, without addition of poly(dI-dC); 3–10, with addition of 50 ng, 100 ng, 200 ng, 400 ng, 800 ng, 1.2 μ g, 2.5 μ g, and 5 μ g, respectively, of poly(dI-dC) in the binding reaction mixture. (B) Competition analysis of binding to the 245-bp cardiac α -actin promoter. Lanes: 1, without additional competitor DNA; 2, with a 50-fold molar excess of the unlabeled 245-bp fragment; 3 and 4, with 10- and 50-fold molar excesses, respectively, of an unlabeled 130-bp fragment (−177 to −48). (C) Electrophoretic mobility-shift assay of the 5'-end-labeled 130-bp fragment (−177 to −48) with nuclear extract from Ltk[−] cells. Lanes 1–10 contain otherwise the same components in the binding reaction mixture as in A.

DNA from the vector or the β -globin gene promoter. Two cardiac α -actin gene promoter regions, one from -177 to -136 and a second from -113 to -84, contain sequences that compete for putative positive regulatory factors. They correspond to the cis regions required for muscle-specific transcriptional stimulation (4) and the sequences conserved among the cardiac α -actin genes of mouse, chicken, and human as shown in Fig. 1 (3). Therefore, the two regions may be responsible for the muscle-specific transcriptional activity of the cardiac α -actin gene through a mechanism that involves binding to them of a positive trans-acting factor in myogenic C2 cells.

Our experiments did not detect a negative regulatory region within the 177-bp promoter segment. We cannot rule out the presence of such a negative region or additional positive regulatory regions, however, because our *in vivo* competition assay may fail to detect the binding of factors present in abundant, nonlimiting quantity in C2 cells. Indeed, our assay failed to detect the TATA box binding proteins, known to exist abundantly (14).

The two positive factor-binding regions each contain a core CArG box, which is found in the promoter regions of several muscle-associated genes (3). The results of DNA mutations in this sequence showed that each CArG sequence plays an important role in tissue-specific expression (4). Therefore, the CArG sequence seems to work as a binding site or as part of a binding site for the positive trans-acting factor or set of factors. Indeed, our data and the preliminary reports of others (21) show that proteins in C2 cells or other muscle cells specifically bind to the CArG box sequences by the gel mobility-shift assay. Although we find a 19-bp consensus sequence that includes the CArG box (4), it is not yet clear to what extent sequences surrounding each CArG box take part in such factor binding. As discussed previously (4), the apparently different factor-binding affinities for the two regions may come from differences of the surrounding sequences or the apparent degeneracy of the A+T-rich core sequence in the CArG box. An alternative explanation for this difference is that the first region may interact with one or more additional positive factors that do not interact with the other region including the second CArG box. However, since the second binding region can compete efficiently for the trans-acting factors binding to the first one, even if such an additional positive factor exists in C2 cells, it does not work by itself but might bind to the appropriate site in conjunction with CArG box-binding factors.

The human skeletal α -actin gene, which is coexpressed with the cardiac α -actin gene in an early stage of muscle development (13, 22), has a region including a CArG box (-98 to -88) as an integral transcription-modulating cis-acting element (23). Furthermore, DNA fragments of the

cardiac α -actin gene promoter that include the CArG box repressed expression of reporter genes driven by the skeletal α -actin gene promoter (G. Muscat, T.M., and L.K., unpublished data). Therefore, the CArG box-binding factors may be used commonly by other muscle-specific genes whose promoters contain CArG boxes. If so, these factors would modulate tissue-specific expression of a series of genes during myogenesis, once the genes were activated.

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