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A REPRESSOR REGION IN THE HUMAN β-MYOSIN HEAVY CHAIN GENE THAT HAS A PARTIAL POSITION DEPENDENCY

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SUMMARY: Expression of the human β-myosin heavy chain (βMHC) gene was studied by transient assay in culture and in situ by direct injection of plasmids into adult rat hearts. In this report we describe a unique repressor region located -326/-309 (5'-TTGGTGGTCGTGGTCAGT-3') of the human βMHC gene that is conserved among the rat, rabbit, and human βMHC genes. This sequence conferred repression onto heterologous promoters when the sequence was located 5' but not 3' to the promoters. This partial positional dependency suggests that the factor may act by limiting the binding of enhancers, located more proximally, to their DNA binding sites.

The MHC isoforms are major determinants of the muscle's functional characteristics and are sensitive to a variety of hormonal and environmental stimuli (1). One of these, βMHC, is expressed in cardiac and skeletal muscle. Studies of the βMHC 5'-flanking sequences in muscle cell lines or primary cardiomyocytes have identified multiple cis-regulatory sequences including both positive and negative elements (2-5). We have previously characterized the βf1 binding region, a strong positive element in the human βMHC located 300 bp from the start of transcription (2). Others have reported a similar element and suggested that this it functions cooperatively with another

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The abbreviations used are: bp, basepair(s); CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain; STI, soybean trypsin inhibitor; T_3, 3,5,3'-triiodo-L-thyronine.

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positive element located about 200 bp from the start of transcription (3,4). Deletion analysis also suggests the presence of a negative element located immediately 5' to these elements (2).

The mechanisms of negative regulation of transcription are less well understood than those of transcriptional activation. Several hypotheses have been advanced; including competition for binding sites, direct influence on the transcriptional complex, and protein-protein interactions which negate the influence of transcriptional activators whether the proteins are in contact with the DNA ("quench") or not ("squelch") (6). Currently, it is unclear which, if any of these models may apply to control of βMHC transcription.

In the present study, we have used constructs containing the human βMHC 5' flanking region fused to the CAT coding sequences to demonstrate the presence of a unique negative element which is functional both in culture and in situ. The region is located immediately 5' to the strong transcriptional activator and may block the site. Binding of the enhancer/promoter trans-factors as a function of the presence of the repressor may determine the overall level of expression of human βMHC gene.

METHODS

Deletion constructs and site specific mutations were made as described previously (2). In each case, the deletions were verified by sequencing the double strand plasmid. Complementary single strand oligonucleotides containing human βMHC sequence from -326/-309 and -317/-297 were used to produce double strand DNA for insertion into the BglII and BamHI sites of pCAT-Promoter Vector (Promega, Madison, WI) which contains the SV40 promoter, as well as the BamHI site of pBL2CAT which contains the thymidine kinase promoter (7).

Heart cells were prepared and electroporated as previously described (2). CV1, HeLa, and C2C12 myoblasts were propagated in DMEM media containing 10% fetal calf serum and transfected by the $CaPO_4$ method using 5μg reporter construct plus 4.0 μg M13 as carrier DNA and 1.0 μg RSVβgal or CMVβgal to determine transfection efficiency (8). For the C2C12 cells the media was changed to DMEM/10% horse serum to initiate differentiation. CAT activity was determined by the method of Gorman et al. and expressed as a function the promoterless parent plasmid (7,9,10). β-galactosidase activity was determined using the method of Eustice et al (11).

For the in situ transfections, 100μg of reporter and 20μg pSV2luciferase was injected into the left ventricle following exposure of the heart by a left thoracotamy. Animals were sacrificed one week after surgery. The hearts were homogenized on ice using the following buffer: 250 mM Tris-Cl pH 7.5, 1 mM DTT, 1mM Benzamidine, 10 μg/ml STI, 10 μg/ml Pepstatin A, 10

μg/ml leupeptin. Luciferase activity was determined by single photon monitoring using the Promega Luciferase Kit (Promega, Madison WI).

RESULTS

We have previously demonstrated several functionally important positive elements located within the 5' flanking region of the βMHC gene (2). Whereas a human βMHC/CAT construct extending to -300 bp from the start of transcription was expressed more than 150 fold above background, constructs extending to -314 and -332 were only expressed 16 and 3 fold above background, respectively (2). DNase "footprint" analysis demonstrated a protected region from approximately -315 to -300 (2). However, full repression requires additional upstream sequence and appears to be complete in the -332 construct. Similar results were obtained using rat βMHC/CAT constructs (data not shown).

A series of site specific mutations were made in the pβMHC468 or pβMHC332 constructs. As shown in Figure 1, mutations centered about the -320 position (pβX322 and pβM322) increased expression about five fold over the parent clone. Singularly, the mutated clones were expressed at a level comparable to that of the pβMHC314 construct. The effect of the double mutation (pβM322/315) was additive suggesting that the DNA binding region was disrupted to a greater extent. Site specific mutations centered about -305 (pβX305) and -289 (pβX289) positions increased expression only two fold.

Independence of the negative element region was examined by cloning the -326/-309 or -317/-297 βMHC regions into heterologous

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      330       320       310       300       290       280       270
       |         |         |         |         |         |         |          FOLD
TGCCTGTGGTCTTGGTGGTCGTGGTCAGTTCCCTCTCCTGCCAGCTGTGGAATGTGAGGCCTGGCCTG      ACTIVATION

------------------------------------------------------------ pβMHC468    2.5±0.4
------------CA-AT------------------------------------------ pβX322     12.0±1.0
----------------------------GTT-A------------------------- pβX305      5.5±0.4
-------------------------------------------A-ATCT----------- pβX289      5.6±0.9

------------------------------------------------------------ pβMHC335    3.5±0.2
-----------CA-AT------------------------------------------ pβM322      15.0±1.1
---------------G---CA------------------------------------- pβM315      11.3±1.1
-----------CA-ATG---CA------------------------------------ pβM322/315  26.9±2.7
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Figure 1. Site specific mutations of the human βMHC gene. The nucleotide substitutions are noted directly below the wild type sequence. The "pβX" constructs are based on the pHβMHC468 clone, while the "pβM" constructs are based on the pHβMHC332 clone. 30μg of reporter plasmid was used in each transfection. The data are expressed as a function of pKSVOCAT activity and are the mean±S.E.

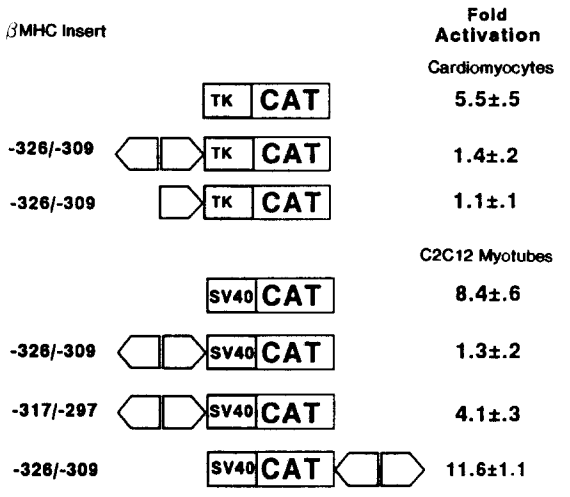

Figure 2. Effect of the repressor region on heterologous promoters. βMHC 5' flanking region was cloned into CAT expression plasmids under the control of the thymidine kinase promoter or the SV40 promoter. Arrows indicate the position, copy number, and orientation of the βMHC sequence in the expression plasmid. Plasmids were transfected into either rat fetal cardiomyocytes (20μg reporter plasmid) or the C2C12 skeletal muscle cell line (5μg reporter plasmid). The data are expressed as a function of the promoterless cassette and are the mean±S.E.

expression vectors containing the thymidine kinase or SV40 promoter. Constructs were transfected into myocardiocytes or C2C12 myotubes. As shown in Figure 2, when oriented as a single copy or as an inverted repeat, the βMHC -326/-309 sequence decreased expression to near background levels. In contrast, the βMHC-317/-297 region decreased expression approximately 50%. More importantly, the βMHC -326/-309 sequence inhibited expression only when located 5' but not 3' to the heterologous promoter.

When transfected into the murine skeletal muscle cell line C2C12, pβMHC332 was expressed only 30% that of pβMHC308. In contrast, as shown in Figure 3A, in neither the HeLa cells or the CV1 cells was repression evident. Experiments in CV1 cells indicated that the heterologous promoters were not inhibited by the presence of the negative element (-326/-309pCAT-Promoter) (data not shown).

The in situ transfection protocols more recently developed have had greater success in muscle compared to other organ systems (12). Adult rats hearts were studied. As shown in Figure 3B, the pβMHC332 was expressed only 6% that of pβMHC308.

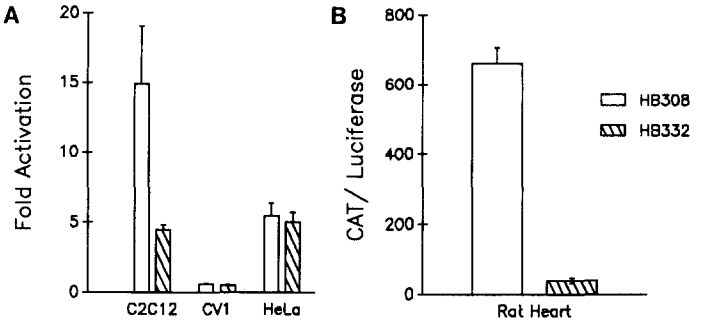

Figure 3. Expression of pβMHC308 and pβMHC332 in situ and in different cell lines. A: C2C12 myotubes, CV1, and HeLa cells were transfected by the $CaPO_4$ method and transfection efficiency determined by β-galactosidase expression. The data are expressed as a function of the promoterless cassette and are the mean±S.E. B: In Situ expression of the human βMHC gene in rat hearts. pβMHC308 or pβMHC332 and pSV2-Luciferase were injected into the left ventricle as described in Methods. The data are expressed as a function of the ratio of CAT to Luciferase activity. Each data point is the mean±S.E.

Qualitatively similar results were obtained in experiments using rabbits (data not shown).

DISCUSSION

Immediately 5' to the βF1 binding region, at positions -326 to -309 is the sequence 5'-TTGGTGGTCGTGGTCAGT-3', which is conserved among the human, rat, and rabbit βMHC genes. In the rat βMHC gene, the GGT motif is continued on the opposite strand from -306 to -298. The region contains a sequence specific negative cis-element that binds a repressor(s) that is active in situ and may be muscle-specific. DNase footprint analysis of this region demonstrated a protected area did not overlap the βf1 region (2,3). The region -316/-312 is similar to the $TGGN_7CCA$ consensus sequence for the NF1/CTF class of DNA binding proteins, which bind to half sites of TGG repeats (13). The region is also somewhat similar to the Human LDL SRE and HMG-CoA reductase repressor elements (13,14). Both are influenced by CNBP, a regulatory protein that may transduce sterol inhibition.

The negative element inhibited expression of vectors under the control of three different promoters. More importantly, the negative element was functional when located 5' but not 3' to the promoters. In each case, the negative element did not overlap the different enhancer/promoter elements and did not compete for the same site. Although speculative, the repressor factor(s) may have limited the binding of enhancers to their respective cis-

elements. This idea is supported by comparing the results of pβX289 with pX2 (2). Both constructs had mutations in the βf1 binding region but the additional 5' flanking sequence prevented the remarkable fall in activity in pβX289 that the pX2 construct demonstrated. This suggests that the βf1 site may have been blocked by trans-factors binding to upstream sequence (2). Alternatively, the repressor may have directly inhibited the transcriptional complex. However, this latter explanation seems less likely since no inhibition was observed when the repressor region was located 3' to the promoter elements (Figure 2). Shimizu et al have suggested that the βf1 and the AP2/NFe-like regions may act synergistically to increase expression (4). If this is true then limiting access to either site will strongly influence constitutive expression.

We have identified a negative element that is located 5' to the three enhancer/promoter regions that regulate constitutive expression of the βMHC gene in the myocardium and skeletal muscle. In the human βMHC gene, the region is located approximately -332 to -300 from the start of transcription. The cis-element contains a GGT motif which is dissimilar to other known repressors and to date no factor has been identified that specifically regulates expression through this site. The cohere trans-acting factor(s) may be muscle specific since the region appears functional only in muscle tissue or cell lines. The region transduces its effect independent of promoter type when located 5' to the promoter elements. Collectively, the data is consistent with the concept, that a site dependent trans-acting factor(s) may act by blocking the binding of transcriptional activators in the βMHC 5'-flanking region. This may in part regulate expression of the βMHC gene.

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