Sequence Elements Required for Transcriptional Activity of the Human Myoglobin Promoter in Intact Myocardium

Rhonda Bassel-Duby, Christian M. Grohe, Michael E. Jessen, William J. Parsons, James A. Richardson, Robert Chao, Jayson Grayson, W. Steves Ring, R. Sanders Williams

Myoglobin is a cytosolic hemoprotein that is abundant in adult cardiac myocytes and oxidative skeletal muscle fibers, in which it probably functions to augment delivery of oxygen for mitochondrial respiration during heavy contractile work. The myoglobin gene is transcriptionally regulated during muscle development and in response to physiological signals generated by muscle activity. Previous studies from our laboratory have defined discrete DNA sequence elements within a 2-kb segment of the 5' flanking region of the human myoglobin gene that are required for transcriptional activation during differentiation of skeletal myogenic cells in tissue culture. In transgenic mice, this 2-kb region is transcriptionally active in cardiac and skeletal myocytes exclusively, but specific sequence elements necessary for transcriptional activity in cardiomyocytes have not been characterized previously.

The goal of this project, therefore, was to identify cis-acting elements from the human myoglobin gene that are required for transcriptional activity in cardiac myocytes within the intact heart. We observed that, in transgenic mice, only 380 bp of the proximal 5' flanking region of the human myoglobin gene is required to recapitulate the pattern of expression of the endogenous mouse myoglobin gene. This same region was sufficient to drive expression of a reporter gene after plasmid injection into the myocardium of adult rats. This technique was used further to map transcriptional control elements responsible for cardiac expression of myoglobin at higher resolution. The results indicate that two sequence elements—a cytosine-rich (CCAC-box) motif and an A+T-rich (A/T) motif—that are conserved in other mammalian myoglobin genes are essential for function of the human myoglobin promoter within the myocardium.

Materials and Methods

Plasmds

The simian virus 40 (SV40) early promoter was used to drive expression of the chloramphenicol acetyltransferase gene (pSVCAT) and the β-galactosidase gene (pSV40lacZ). The plasmid pGUPPA.8 (general use plasmid with upstream polyadenylation sequence) was constructed by inserting a synthetic oligonucleotide that reproduces the proximal 58 bp of the human hsp70 promoter upstream from the firefly luciferase gene, carried in Bluescript KS+ (Stratagene Inc, La Jolla, Calif). The pGUPPA.8 construct also includes an SV40 polyadenylation signal located 5' to the promoter region to eliminate background expression of luciferase resulting from cryptic transcriptional initiation sites within the prokaryotic vector sequences. Plasmid pRSV-LUC uses the Rous sarcoma virus (RSV) long terminal repeat to drive expression of the firefly luciferase gene. Plasmid pCK9 was constructed by insertion of the

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Drs Bassel-Duby and Grohe contributed equally to the study.

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The gene.
The
Resources
Center
pGEM-7Z
vector
(pCCACmut,
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were
Oocytes
and
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Evolutionary
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The
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A/T-rich
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[1-160
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and
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box
[-204
to
-226] relative
to
the
cap
site
are
shown.
Evolutionary
conservation
of
these
motifs
is
illustrated
by
comparison
to
upstream
sequences
of
the
mouse,
seed,17
and
human2
myoglobin
genes.
Nonconserved
bases
and
base
substitutions
that
generate
mutant
forms
of
the
myoglobin
promoter
(CCACmut,
A/Tmut,
or
E1+2mut)
are
highlighted.

301-bp upstream enhancer region from the mouse creatine kinase gene15 into pGUPPA.8
The
plasmid
p380-LUC
was
constructed
by
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an
upstream
fragment
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to
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human
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1)’s
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blurred
in BlueScript
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This
construction,
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pGUPPA.8,
also
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an
SV40
polyadenylation
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the
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of
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of
the
myoglobin
sequence.
Nucleotide
substitutions,
directed
at
disrupting
sequence
motifs
within
the
CCAC
box,
A/T
motif,
and
E-box
sites,
were
introduced
into
p380-
LUC
using
oligonucleotide-directed
mutagenesis
as
described.8
The
sequences
of
the
resulting
mutant
clones
(pCCACmut,
pEmu1+2,
and
pAa/Tmut)
were
confirmed
by
restriction
mapping
and
DNA
sequencing.
The
DNA
segment
used
for
transgenic
animal
experiments
contained
the
myoglobin-luciferase
fusion
present
in
p380-LUC
but
was
engineered
in
the
pGEM-7Z
vector
(Promega
Corp,
Madison,
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include
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SV40
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and
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region.
Oocytes
were
injected
with
linear
DNA
purified
as
an
Aat II–Nsi I
fragment.

Mouse Oocyte Injections and Embryo Analysis
Mice
cos
strain
FVB/N-HSD)
were
housed
in
the
Animal
Resources
Center
of
the
University
of
Texas
Southwest-
ern
Medical
Center
at
Dallas,
and
experimental
protocols
were
approved
by
the
Institutional
Review
Board
for
Animal
Research.
After
microinjection
of
DNA,
fertil-
ized
oocytes
were
implanted
into
pseudopregnant
females.
Embryos
were
examined
at
a
gestational
age
of
18.5
days
by
Southern
analysis
(not
shown)
to
identify
animals
that
had
integrated
the
transgene
into
chromo-

somal
DNA.
Tissues
of
all
transgene-positive
animals
and
two
transgene-negative
littermates
were
fixed
overnight
at
4°C
in
0.1
M
phosphate-buffered
saline
and
4% paraformaldehyde,
ph
7.5,
then
dehydrated,
cleared,
paraffin-embedded,
sectioned,
mounted,
and
hybridized
toradiolabeled
probes,
as
described
previously.7
Hybrid-
ization
probes
were
labeled
during
in
vitro
transcription
in
the
presence
of
32S-UTP.
Luciferase
mRNA
was
detected
using
a
540-nt
antisense
RNA
transcript
complementary
to
firefly
luciferase
mRNA
(+100
to
+63913).
Endogenous
mouse
myoglobin
mRNA
was
detected
with
a
503-nt
antisense
probe
complementary
to
exon
3
of
the
mouse
myoglobin
gene,7
and
the
corresponding
sense
strand
served
as
a
control
for
nonspecific
binding.

Heart Injection Protocol

Rats
(Sprague-Dawley)
were
housed
in
the
Animal
Resources
Center
of
the
University
of
Texas
Southwest-
ern
Medical
Center
at
Dallas,
and
experimental
pro-
tocols
were
approved
by
the
Institutional
Review
Board
for
Animal
Research.
In
initial
experiments
8-week-old
male
Sprague-Dawley
rats
were
anesthetized
and
intu-
bated
as
described
previously.9
In
later
experiments,
rats
were
anesthetized
with
methoxyflurane
inhalation.
After
left
lateral
thoracotomy
and
exposure
of
the
left
ventricle,
100
µL
of
buffer
containing
plasmid
DNA
dissolved
in
5% sucrose,
10
mM
Tris,
ph
7.4,
and
1
mM
EDTA
was
injected
into
the
depo
table
portion
of
the
left
ventricular
wall
using
a
27-gauge
needle.
Animals
were
allowed
to
recover
and
were
euthanized
4
days
later
by
pentobarbital
injection
(120
mg/kg
IP).

Injections
were
performed
with
100
µg
test
plasmid
DNA
carrying
the
luciferase
gene
controlled
by
various
promoters.
Experiments
comparing
activity
of
mutant
myoglobin
promoter
sequences
to
that
of
the
wild-type
sequence
present
in
pMb380-LUC
were
performed
in
two
ways:
(1)
with
cotransfection
of
50
µg
pSVCAT
as
an
internal
control
for
variations
in
the
efficiency
of
DNA
uptake
into
cardiomyocytes
and
(2)
without
cotransfection
of
pSVCAT,
to
avoid
potential
con-
 founding
effects
of
competition
by
the
SV40
promoter
for
factors
involved
in
transcriptional
activity
of
the
myoglobin
promoter.

The
apical
tissue
of
each
heart
was
resected,
rinsed
in
Dulbecco’s
phosphate-buffered
saline
at
0°C,
and
weighed.
Ventricular
myocardium
(300
mg)
was
minced
with
a
scalpel
and
suspended
in
500
µL
ice-cold
homogenization
buffer
containing
(mM)
po-
tassium
phosphate,
100
(pH
7.8);
magnesium
sulfate,
15;
and
dithiothreitol
1.
The
tissue
was
homogenized
for
30
seconds
(Polytron
Kinematica,
Switzerland),
and
10
µL
octyl-phenoxy-polyethoxyethanol
(Sigma
Chemical
Co,
St
Louis,
Mo) was
added.
After
centrif-
ugation
at
3000g
for
10
minutes
at
4°C,
the
supernatant
was
removed
and
held
on
ice.
Enzyme Assays

To determine luciferase activity in myocardial cell lysates, aliquots containing 50 μL of each supernatant were mixed with 250 μL assay buffer, and light emission was quantitated in a Beckman LS 5000 TA liquid scintillation counter after addition of 100 μL of 1 mM luciferin (Sigma) dissolved in assay buffer. Fifty microliters of each supernatant also was assayed for chloramphenicol acetyltransferase activity, as described previously.18

Preparation of Nuclear Proteins From Isolated Cardiomyocytes

Hearts were excised from 1 to 2-day-old Sprague-Dawley rats. Cardiomyocytes were dispersed with collagenase II (Worthington Biochemical Corp, Freehold, NJ) and centrifuged through a discontinuous Percoll gradient as previously described. The myocytes were plated at 1.5 × 10^6 cells per 60-mm Falcon dish in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotic (3 μg/mL gentamicin).

Nuclear extracts were prepared by washing the cell monolayers three times with isotonic buffered saline followed by addition to each 60-mm plate of cells 0.4 mL lysis buffer (20 mM HEPES [pH 7.6], 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 100 μg/mL aprotinin). The cells were harvested by scraping the plates with a rubber policeman, placing the cellular suspension in a microfuge tube, and centrifuging for 1 minute at 2000 rpm at 4°C. The pellet was resuspended in nuclear extraction buffer (lysis buffer with 500 mM NaCl), rocked for 1 hour at 4°C, and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at 4°C. Protein concentration was determined by the Bio-Rad protein assay based on the Bradford20 dye-binding procedure (Bio-Rad Laboratories, Richmond, Calif).

Gel Mobility Shift Assays

Mobility shift reactions were prepared in a final volume of 12 μL by combining 1 ng of end-labeled double-stranded DNA (≈10^8 cpm) with nuclear extract (≈3 μg) and 1 μg poly (dl-dC) (Boehringer Mannheim Biochemicals, Indianapolis, Ind) in a binding buffer containing 25 mM HEPES (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol, and 0.2% Nonidet P-40. Unlabeled competitor oligonucleotides (100 ng) were included in some reactions as indicated in Fig 5. After 20 minutes of incubation at room temperature, the reactions were loaded onto a 4% native polyacrylamide gel equilibrated in 45 mM Tris–45 mM boric acid–1 mM EDTA. After electrophoresis at 10 V/cm for 40 minutes, the gels were dried and exposed to Hyperfile-ECL (Amersharm Corp, Arlington Heights, Ill) for 20 hours. The sequences of oligonucleotide probes and competitors included the following: CCAC (sense), 5'-GATCACGCAACACCCACCCACCCCTGGTGG-3'; CCAC (antisense), 5'-GATCCAACAGGGGTTGGGTTGTTGCGT-3'; CCACmut (sense), 5'-GGTCACAACACCCGGTGACCTGTCG-3'; CCACmut (antisense), 5'-GTGCT-

CAGGCCACAGGTACGGGTTGTTGTG-3'; A/T (sense), 5'-GATCCTGCTAAATTAGCTCC-3'; and A/T (antisense), 5'-GATCGGGAAGCTATTTAAGGCAG-3'.

Data Analysis

Data concerning luciferase activity are presented as counts per minute per gram wet weight. In Fig 3, results are corrected for recovery of chloramphenicol acetyltransferase activity in each sample, which ranged from 9770 to 19,230 cpm/50 μL cell extract (mean, 12,500 cpm; background, 3500 cpm). Luciferase activity expressed from constructs bearing mutations in myoglobin promoter sequences was compared with that expressed after transfection of p380-LUC by Student's t test for unpaired variables, with correction for multiple comparisons.

Results

Transcriptional Activity of the Human Myoglobin Promoter in Transgenic Mouse Embryos

Of 31 offspring from 4 females, 6 animals had integrated the transgene, as assessed by Southern analysis (not shown). Luciferase mRNA was detected by in situ hybridization in cardiac ventricles from 5 of the 6 transgenic animals. The expression of luciferase under the control of the human myoglobin upstream region between −373 and +7 was limited to cell types that also expressed the endogenous mouse myoglobin gene. As shown in Fig 2, binding of the myoglobin and luciferase antisense RNA probes was abundant within the left ventricular cardiac wall but was present only at background levels in atrial myocytes, nerve, pericardium, or lung parenchymal cells. Luciferase and myoglobin mRNA could be detected in skeletal myocytes of transgenic embryos, but only background binding of either probe was detected in nonmuscle tissues (eg, liver and brain) of any animal (not shown). Similar results were observed in the other 4 transgenic animals that expressed the transgene. Luciferase mRNA could not be detected in littersmates that had failed to integrate the transgene. A noncomplementary myoglobin (sense) probe demonstrated only low background binding that was uniform across all tissues (not shown).

Thus, the proximal upstream region (−373 to +7) from the human myoglobin gene is sufficient to direct expression of a luciferase reporter gene in cardiac muscle of transgenic mouse embryos in a manner that parallels the expression of the endogenous mouse myoglobin gene. This pattern of myoglobin expression changes after birth with respect to two features: transcription (1) is activated in the atria and (2) becomes more homogenous across the ventricular wall. We have reported previously that a larger upstream fragment (−2038 to +7) from the human myoglobin gene is regulated in a similar manner in transgenic mice, but the present experiment was not designed to monitor these postnatal events.

Transcriptional Activity of the Human Myoglobin Promoter in Left Ventricles of Adult Rats

The same upstream region of the human myoglobin gene (−373 to +7) tested in transgenic mice was analyzed for transcriptional activity by direct injection of plasmid DNA into the left ventricular wall of adult
rats. In pilot experiments (not shown), the expression of luciferase activity following introduction of pMb380-LUC was comparable to that of pRSV-LUC and pCK9 and consistently greater than that of pGUPPA.8. Considerable variability in the expression of luciferase activity was observed with all three of the active promoter constructs, indicating the importance of an internal standard such as pSVCAT that was included in the subsequent analysis of mutations within the myoglobin promoter. Histological sections of myocardium stained for β-galactosidase activity after transfection of pSV40lacZ (not shown) confirmed reports of others that transgenes are expressed in cardiomyocytes but not in other cell types after plasmid injection into the myocardial wall.

Mutational Analysis of the Myoglobin Promoter Region in the Intact Rat Heart

We compared the activity of the wild-type myoglobin promoter in pMb380-LUC to that of constructs in which nucleotide substitutions were introduced into the CCAC box (−226 to −205), an A/T element–like binding site (−169 to −160), or both of two CANNTG (E-box) motifs present within this region (−176 to −148). All three of these sequences are conserved in myoglobin genes from other mammalian species (Fig 1). Previous studies demonstrated that the CCAC box and A/T motifs are required for transcriptional activity of the human myoglobin promoter in sol8 cells, a skeletal myogenic cell line. A requirement for these sequence elements for transcription in skeletal muscle was also seen in primary cultures of chick embryo myotubes (R. Bassel-Duby, Z. German, and R.S. Williams, unpublished data). As shown in Fig 3, disruption of the CCAC box or A/T motif severely impaired transcription of the myoglobin promoter in the intact rat heart, whereas mutations that disrupted the E-box motifs had a less profound effect in the cardiac muscle background.

To determine whether results of the mutational analysis were influenced by competition between the SV40 and myoglobin promoters for limiting concentrations of certain transcription factors, some of these experiments were repeated in the absence of cotransfected pSVCAT. In this separate set of experiments, disruption of the CCAC box (n=9) reduced expression of luciferase to less than 5% of that produced by transfection of p380-LUC (P<.01), whereas mutations within both E-box motifs (n=6) had a less striking effect (39% of p380-LUC, P=NS). These results are similar to the set of experiments performed with cotransfection of
wall bind in vitro to nuclear protein factors isolated from cardiomyocytes. Nucleotide substitutions within these sequence elements that disrupt promoter function also disrupt binding of nuclear proteins, as assessed by competitive binding studies.

**Discussion**

Myoglobin is a member of a set of genes that are expressed in both skeletal and cardiac myocytes but transcriptionally silent in nonmuscle cells. The molecular mechanisms that govern such restricted expression may include cis-acting transcriptional control elements and transcription factors that are common to both lineages of striated muscle or are unique to one and not the other cell lineage. For example, myogenic determination genes from the basic helix-loop-helix family occupy a pivotal role in skeletal myogenesis but cannot be detected in cardiomyocytes. The transcription factors that serve an analogous function during cardiac development remain to be identified.

We have previously studied the regulation of the myoglobin gene in skeletal muscle in some detail. Previous studies of the mechanisms of its expression in cardiac muscle are less extensive, but experiments in transgenic mice demonstrated that a 2-kb upstream fragment (−2038 to +7) of the human myoglobin gene is sufficient to direct expression of a reporter gene selectively in skeletal and cardiac muscle of developing mouse embryos. This implies that, unlike certain other genes, muscle-specific enhancer regions that control expression of myoglobin in cardiomyocytes reside within the same genomic segment as those that govern expression in skeletal muscle. The present study was designed to identify at higher resolution cis-acting transcriptional control elements required for transcription of the myoglobin gene in cardiomyocytes. For this purpose, we combined additional studies in transgenic animals with the recently developed technique of direct plasmid injection into the myocardium.

The data indicate that a limited segment (−373 to +7) of the upstream region of the human myoglobin gene is sufficient to direct transcription of a reporter gene in the heart. This conclusion is supported both by
analysis of transgenic mice and of rat myocardium following somatic cell gene transfer. Analysis of base substitution mutants indicates that cardiac transcription requires at least two sequence motifs within this region: a CCAC box, and an A/T motif. Consensus E-box motifs within the myoglobin promoter appear to have lesser importance in cardiac muscle, as we have observed previously in skeletal myocytes.6

The CCAC-box sequence is conserved in the promoter region of myoglobin genes from other mammalian species (Fig 1), and similar sequences are present within transcriptional control regions of other genes that are expressed exclusively in skeletal and cardiac muscle.23 Results from the present study indicate that cardiomyocytes express nuclear proteins that bind to the CCAC element in a sequence-specific manner. The identity of these cardiomyocyte proteins remains to be determined definitively but may be related to CCAC-box binding proteins we have characterized from skeletal myocytes in other studies. These include a 40-kD protein purified from so8 myotubes and a less abundant 89-kD protein identified by screening bacterial expression libraries with a radiolabeled CCAC sequence as probe. Antibodies raised against this latter protein cross-react with a protein of similar size in Western blots of nuclear proteins from isolated cardiomyocytes (R. Bassel-Duby, M. Hernandez, and R.S. Williams, unpublished data).

Cardiomyocytes also express nuclear protein factors that recognize the A/T motif from the myoglobin upstream region, as shown in the present study. In other investigations (not shown), gel mobility shift assays indicate that this region is capable of binding MEF223,24 but at a lower affinity than a consensus MEF2 element from the mouse creatine kinase gene. This region also binds specifically to other factors present in muscle extracts that can be distinguished from MEF2 by competitive binding assays (J. Grayson, R. Bassel-Duby, and R.S. Williams, unpublished data). Determination of the identity of the factor(s) that recognizes this functionally important control region in cardiomyocytes also is a major goal of ongoing investigations.

E-box motifs are evolutionarily conserved within myoglobin genes of several species. We suspect that binding of basic helix-loop-helix proteins to these sites may have a role in transcriptional activation of the myoglobin gene during skeletal or cardiac muscle development, but this function appears to be largely redundant to that of other factors under the conditions used for analysis in the present study and previous studies6,7 of the myoglobin promoter.

We conclude from the present study that combinatorial interactions between the cognate DNA binding factors that recognize CCAC-box and A/T motifs within the human myoglobin upstream region are necessary for transcriptional activity in cardiac muscle. Further characterization of these factors and elucidation of the nature of this functional interaction in molecular terms should increase our understanding of the control of gene expression in cardiac myocytes and of the developmental biology of the heart.

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References


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