Nuclear respiratory factors NRF1 and NRF2 regulate the expression of nuclear genes encoding heme biosynthetic enzymes, proteins required for mitochondrial genome transcription and protein import, and numerous respiratory chain subunits. NRFs thereby coordinate the expression of nuclear and mitochondrial genes relevant to mitochondrial biogenesis and respiration. Only two of the nuclear-encoded respiratory chain subunits have evolutionarily conserved tissue-specific forms: the cytochrome c oxidase (COX) subunits VIa and VIIa heart/muscle (H) and ubiquitous (L) isoforms. We used genome comparisons to conclude that the pro-matrix transcription factor, NRF1 regulates the expression of nuclear and mitochondrial genes required for mitochondrial genome transcription and protein import, and numerous respiratory chain subunits. NRFs thereby coordinate the expression of nuclear and mitochondrial genes relevant to mitochondrial biogenesis and respiration. Only two of the nuclear-encoded respiratory chain subunits have evolutionarily conserved tissue-specific forms: the cytochrome c oxidase (COX) subunits VIa and VIIa heart/muscle (H) and ubiquitous (L) isoforms. We used genome comparisons to conclude that the promoter regions of COX6A2 and COX7A2 lack NRF sites but have conserved myocyte enhancer factor 2 (MEF2) elements. We show that MEF2A mRNA is induced with forced expression of NRF1 and that the MEF2A 5′-regulatory region contains an evolutionarily conserved canonical element that binds endogenous NRF1 in chromatin immunoprecipitation (ChIP) assays. NRF1 regulates MEF2A promoter-reporters according to overexpression, RNA interference underexpression, and promoter element mutation studies. As there are four mammalian MEF2 isoforms, we used an isoform-specific antibody in ChIP to confirm MEF2A binding to the COX6A2 promoter. These findings support a role for MEF2A as an intermediary in coordinating respiratory chain subunit expression in heart and muscle through a NRF1 → MEF2A → COX6A2 transcriptional cascade. MEF2A also bound the MEF2A and PPARGCA1 promoters in ChIP, placing it within a feedback loop with PGC1α in controlling NRF1 activity. Interruption of this cascade and loop may account for striated muscle mitochondrial defects in mef2a null mice. Our findings also account for the previously described indirect regulation by NRF1 of other MEF2 targets in muscle such as GLUT4.

The electron transport chain (ETC) consists of four multi-subunit enzyme complexes within the inner mitochondrial (mito) membrane. These act in concert to transfer electrons from succinate or NADH to molecular oxygen while pumping protons from the matrix to the intermembranous space, establishing the electrochemical gradient required for oxidative phosphorylation (OXPHOS) (1). Nuclear genes encode all of the components of complex II, but the other complexes have subunits encoded by both mito (ETC\textsubscript{mito}) and nuclear (ETC\textsubscript{nucl}) genes (1, 2). Appropriate ETC subunit stoichiometry requires the coordinate expression of genes on the two genomes and an accounting for a variable number of mito genomes per cell (2, 3). This is orchestrated by the nuclear respiratory (transcription) factors, NRF1 and NRF2 (2–5). These structurally unrelated factors, encoded by nuclear genes, regulate the transcription of TFAM, TFB1M, and TFB2M, nuclear genes of the mito transcription factor Tfam (mtTFA) (6) and Tfbm specificity factors (7). Tfam and Tfbm proteins are imported into mito where they direct transcription from both heavy and light strands of mito DNA (mtDNA). These transcriptions are processed to yield the various ETC\textsubscript{mito} mRNAs, as well as rRNAs, tRNAs, and a primer for the RNA-dependent activity of DNA polymerase γ and mtDNA replication. This NRF → [TFAM, TFBM] → ETC\textsubscript{mito} transcriptional cascade functions in parallel with the direct control of promoters of overlapping sets of ETC\textsubscript{nucl} genes by NRF1 and NRF2 (2, 3, 8). As NRFs also regulate nuclear genes encoding complex V (F₁F₅-ATPase) subunits, heme biosynthetic enzymes, and mito protein import machinery (2, 3, 9, 10), they are the central regulators of mito biogenesis and cellular respiration. This is underscored by the mito deficiency and peri-implant lethality of mff1 null mouse embryos (11).

Although the entire ETC can be regarded as functioning as a single unit, the reaction catalyzed by cytochrome c oxidase (COX, complex IV) involves the largest free energy change in the mitochondrial respiratory chain, leading to the formation of ATP.

The abbreviations used are: ETC, electron transport chain; AMPK, 5′-AMP-activated protein kinase; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; COX, cytochrome c oxidase; CYCS, cytochrome c, somatic form; EGW, erect wing; GLUT4, facilitated glucose transporter 4; MEF2, myocyte enhancer factor 2; mito, mitochondrial(on/a/al); NRF, nuclear respiratory factor; OXPHOS, oxidative phosphorylation; PPAR, peroxisome proliferator-activated receptor; PGC1α, PPARγ coactivator 1α; QCPR, quantitative polymerase chain reaction; RPA, ribonuclease protection assay; TSS, transcription start site; ds, double stranded; HEK, human embryonic kidney; GFP, green fluorescent protein; siRNA, small interfering RNA; RNAi, RNA interference; chr, chromosome.
among ETC reactions (1). Subunits I, II, and III that together form the catalytic core of COX are encoded in the mito genome, whereas the remaining 10 subunits serve structural or regulatory functions and are products of nuclear genes. Among ETC enzymes, COX alone has tissue-specific subunit isoforms that are the products of separate nuclear genes. Thus, there are both energy demand (14).

As described (17, 18). Human (RPA) and radiolabeled cRNA probe syntheses were carried out Ambion. Murine tissue and C2C12 and 10T1/2 cell RNA was subcloned into pBluescript, oriented to permit cRNA production and transcription-PCR with primers 5'-GATGCGCAGG-3'

and 5'-TCAC-3'.

Experimental procedures

RNA Analyses—Human tissue RNA was obtained from Ambion. Murine tissue and C2C12 and 10T1/2 cell RNA was isolated as described (17). Ribonuclease protection assays (RPA) and radiolabeled cRNA probe syntheses were carried out as described (17, 18). Human MEF2A and murine mef2a, mef2c, and mef2d cRNA probes have been described (17-19). Templates for other cRNA probes were PCR amplification fragments subcloned into pBluescript, oriented to permit cRNA production from the vector T7 promoter. The 149-bp template for murine mrf1 cRNA used mouse heart RNA and reverse transcription-PCR with primers 5'-cgggacGTCCTCGGGGCA-3'

and 5'-ggcgaggattCTTCCTGTCGGACATC-3'. The underlined letters are restriction sites used in subcloning. The uppercase letters are cDNA sequences lower-case and 5'-GCTCTGGTGCCAGACATC-3'; (MEF2A, 187 bp) 5'-GTGTATCCTAGCAATGCGAC-3' and 5'-AACCCTAGATATGCGCTTCTC-3'; (CYS, 106 bp) 5'-GGTGAAGAGGAGAAGAGCA-3' and 5'-TGTCTGTTGCGGCTGTC-3'; and (ACTB, 233 bp) 5'-GGACTGTCGAGAAGATGG-3' and 5'-AGCCTGTTGGGCCTAG-3'. QPCR primers for murine cDNAs were: (gapdh, 223 bp) 5'-CTGGAGAAACCTGCAAGTA-3' and 5'-TTGCCGCTGCTGTTG-3'; (cycs, 177 bp) 5'-TTCCAGAAGTGTGCAGGG-3' and 5'-CTCCATAATCAATCCAGGATCCGTC-3'; (cox5b, 129 strain heart cDNA and IMAGE clone 1248366, 5'-ggggcgcgtacAGGTACGG-3' and 5'-AATTAATTTGGATGATGACGAG-3'. Mouse mfe2a and mrf1 primer pairs were identical to the respective human MEF2A and NRF1 primers. Real-time PCR results were analyzed using MX-3000 software, and the various mRNA quantities were normalized to the expression of a gene of interest (ACTB or actb). In brief, the fractional difference in the expression of a gene of interest (goi) mRNA in experimental (e) versus control (c) samples was determined using the formula ∆mRNA (arbitrary units) = 2^-[Δgoi - Δactb] , where ∆goi is the difference [e] - [c] in cycle number at critical threshold for the goi and ∆actb is the difference [e] - [c] in cycle number at critical threshold for actb. Fluorescence determinations were also used to establish conditions (cycle number) to retrieve aliquots of parallel reactions for direct visualization of amplon levels by agarose gel electrophoresis. All reported results were repeated in three independent small interfering RNA (siRNA) transfections.

Reporter Plasmids—p5kLuc and the MEF2Ap1-luc and MEF2Ap2-luc deletion series and p1[mMef2]-Luc and p2[pMef2]-Luc have been described (19). tata-Luc (Gift of Grace Gill) has been referred to as E1BLuc (20). p1[1mNrf1]-Luc and p2[2mNrf1]-Luc were created using PCR mutagenesis, exploiting the MEF2A promoter FspI site within the Nrf1 element (TGG\_GCAAGGCGA). Mutations corresponded to the difference [e] - [c] in cycle number at critical threshold for actb. Fluorescence determinations were also used to establish conditions (cycle number) to retrieve aliquots of parallel reactions for direct visualization of amplon levels by agarose gel electrophoresis. All reported results were repeated in three independent small interfering RNA (siRNA) transfections.

NRF1 Regulates MEF2A Expression

Gene encoding the H and L forms of COX VII are sometimes referred to as COXH7A1 and COXH7A2. The underlined letters are restriction sites used in subcloning and 5'-GATGCGCAGG-3'.
NRF1 Regulates MEF2A Expression

**Eukaryotic Expression Vectors**—The NRF1 coding region from Mobility Shift Assays

**C2C12 or HEK 293 cells** were grown to confluence prior to cross-linking and subcloning an RPCI-98-01L01 BAC clone EcoRI fragment containing a transcription factor binding site for the relevant transcription factor. Insert lacking a stop codon from pCDNA-NRF1VP16 with a SbfI site downstream of an SbfI site. pCDNA3-EWG and pCDNA-NRF1VP16 were transferred into FspI- and NcoI-cut S-DraI (m2NRF1), and the NcoI-blunt fragments were substituted into pET-NRF1. pET-NRF1myc was created by substituting a NRF1 coding region was PCR-amplified from IMAGE cDNA clone 30094033 SalI to NotI insert. pCDNA-PGC1α-2/m1MEF2C

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NRF1 Regulates MEF2A Expression

all had 19 bp corresponding to the targeted mRNA and 3’ UU extensions on each strand. Human siRNA sense sequences were: GAPDH, 5’-GUCAACGGAUUGGUGGUAU-3’; NRF1, 5’-GAACCGCCUCAUGUAUUAUU-3’, 5’-UGUGAUAGCCAUCAUGUAU-3’, 5’-CACAUGGCGUGAUCGUAAU-3’, and 5’-GCAUUGCCCUCAUGUAU-3’. Mouse siRNA sequences were: gapdh, 5’-GGUGAAACCGAAGAUAUUUU-3′; nrf1, 5’-GAAGAAGCCCGCCGGAAUAU-3’, 5’-GAUGAAUGCUACUCUGUAU-3’, 5’-UGAAUAAGCCCUCCGUAU-3’, and 5’-CAACAGGGAAAGACGGAAUU-3′. A control siRNA that fails to recognize any human or mouse targets (Qiagen catalog No. 1027280) was used as a negative control. An Alex Fluor 488-labeled negative control ds-siRNA (Qiagen) was used initially to optimize transfection conditions.

Miscellaneous Reagents and Procedures—All plasmid segments derived from PCR were verified by dideoxy sequencing (21). Mouse anti-myc epitope (clone 9E10) was from United States Biologicals, and anti-NRF1 antiserum was the generous gift of Richard Scarpula (Northwestern).

RESULTS

MEF2 Regulatory Elements Are Common to Heart/Muscle-specific Respiratory Chain Subunit Gene Promoters—We examined the distribution of cox6a and cox7a isoform mRNA in adult mouse tissues using RPA to discriminate between the highly similar mRNA sequences. Consistent with previous reports (22), cox6aH was present exclusively in skeletal muscle and heart, whereas cox6aL was expressed in all other tissues examined (Fig. 1). cox7aH was also expressed exclusively in striated muscle, and although cox7aL was highest in non-muscle tissues, it was present in skeletal muscle and quite abundant in heart. cox7aL, a third isoform of this subunit (23), was expressed ubiquitously at a relatively low level. Using 10T1/2 MyoD-ER cells wherein myogenesis is induced by estrogen (24), isoform switching from cox6aL to cox6aH coincided with the appearance of a myosin heavy chain skeletal muscle differentiation marker (not shown).

The COX6A, COX7A, and COX8 (H and L forms of each) are compact genes, each having a single promoter and splicing pattern and one initiation codon within the first exon (8). We compared 5′-flanking region sequences of each of these genes among mammals to identify conserved elements (Fig. 1). Transgenic promoter-reporter studies in mice have shown that MEF2 and E box elements provide heart- and muscle-specific expression of mouse cox6aH (16). The corresponding region of other mammalian COX6A/L genes is highly conserved (25). Specifically, the region has either one canonical MEF2 element or two sites separated by 7–10 bp (supplemental Table S1). COX7A/L and COX8/L gene regulatory regions also have functional MEF2 elements (15, 16). No sequences corresponding to NRF1 or NRF2 elements were detected in COXH promoters. By contrast, both the COX6A/L and COX7A/L promoters have putative or documented NRF1 or NRF2 elements (2, 3, 8), but none has a MEF2 site. Although the COX7A/L gene promoter region has not been functionally characterized, we found NRF1 and MEF2 elements within 1.2 kb of the transcription start site (TSS). No other transcription factor binding sites that are conserved and selectively present in the COXH versus COXL promoters were found.

Figure 1. Tissue-specific COX subunit 6A and 7A isoform genes have conserved promoter elements. cRNA probes (p) specific for murine cox6a and cox7a isoforms were used in RPA with 2 μg of mouse brain (B), heart (H), liver (L), skeletal muscle (M), and testis (T) total RNA. Assays were performed with cRNA panels used probes from C57BL/6J (left) and 129 strain templates (right), which differ at 2 nucleotides. RNA was isolated from 129 (heart) and C57BL/6J (other tissues) mice. The locations of MEF2 (diamonds) and NRF1 (hexagons) elements in the COX subunit gene promoters are shown below each RPA.
NRF1 Regulates MEF2A Expression

MEF2A Promoter 1 Contains a Conserved Canonical NRF1 Element—We mapped the 5′-regulatory region of human MEF2A. The gene has two closely approximated alternative first exons, A1 and A2, and cognate promoters ∼65 kb upstream of the first coding exon 1 (Fig. 3, A and B). A MEF2 element overlies the major promoter 1 TSS (TSS p1, and we have shown that this site provides for MEF2A transcriptional autoregulation (19). Of particular interest to our hypothesis, a canonical NRF1 (2, 4) element is centered at −47 relative to TSS p1 (Fig. 3C and supplemental Fig. S1). Two E boxes and two potential NRF2 sites (2, 5) are located further upstream (Fig. 3B and supplemental Fig. S1). The MEF2A 5′-regulatory region within ∼0.8 kb upstream of TSS p1 is highly conserved among mammals, and the MEF2, NRF, and E box elements are each conserved in sequence and location (supplemental Fig. S1). The proximal promoter 1 location of the putative NRF1 element and its evolutionary conservation strongly suggested functional relevance.

We performed both mobility shift and chromatin immunoprecipitation (ChIP) assays to verify NRF1 binding to the putative MEF2A promoter element. In the former, one specific retarded complex was formed in resolved binding reactions of recombinant epitope-tagged NRF1 (NRF1myc) and a dsDNA probe containing the element (Fig. 3E). This complex was not competed by otherwise analogous probes containing either two (m1NRF1) or four (m2NRF1) substitutions within the GC core repeats of the element (Fig. 3D). The complex was supershifted in reactions containing anti-myc antibody, confirming a recombinant NRF1 component. Specific complexes were also formed with the probe and C2C12 myoblast and myotube nuclear protein extracts, and these were supershifted in resolved reactions containing anti-NRF1 antibody (Fig. 3F).

Endogenous nuclear NRF1 interaction with the MEF2A promoter element was verified using ChIP. PCR primers were designed to amplify the region surrounding TSS p1 that includes the NRF1 and MEF2 elements (Fig. 3B), or a downstream region. Anti-NRF1 antibody specifically co-precipitated the MEF2A promoter fragment from HEK 293 (human) cells (Fig. 3G). Similar findings were obtained in ChIP assays using C2C12 (murine) cells and primers designed to detect the analogous region of mef2a (not shown). We had previously shown that MEF2A factors also bind this promoter region (19). NRF1 is therefore positioned to directly regulate MEF2A transcription and to influence its autoregulation.

Forced NRF1 Expression Induces MEF2A Promoter Activity—To evaluate the influence of the NRF1 element and factor on MEF2A promoter 1 and 2 activities, we used the MEF2A promoter 1 and 2 Luc reporters, respectively (19) (Fig. 4A). In general, target gene promoters are only modestly sensitive to NRF1 overexpression in mammalian cells (30). By contrast, forced expression of NRF1 (or its ortholog, EWG) has been reported to have robust activity in cultured Drosophila cells (20, 30). We therefore began by examining the effects of NRF1 expression on the MEF2A reporters in Drosophila S2 cells. Our focus on the NRF1 element led us to use the S-MEF2A promoter 1-Luc and S-MEF2Ap2-Luc constructs that contain only proximal promoter fragments in order to exclude superfluous upstream enhancers (19). We expressed either human NRF1 or its Drosophila ortholog, EWG, the product of erect wing (20, 31), or a fusion or mutant of either (Fig. 4B) from the Drosophila B-actin promoter. In these studies, MEF2Ap1-Luc activity was induced 17-fold and MEF2Ap2-Luc 25-fold in cells expressing full-length NRF1 (Fig. 4C). Forced expression of EWG gave less potent effects, but an EWG mutant deleted for the N terminus (EWGΔN144) (20) activated both MEF2A promoters to a similar extent as NRF1. These transcription factors gave up to 400-fold stimulation of [NRF1]MEF2A promoter-Luc, a reporter containing 3 copies of the NRF1 site, but had minimal effect on the parent tata-Luc control. The MEF2A promoters are highly responsive to forced expression of NRF1 in the nuclear milieu provided by this cell type.

To evaluate MEF2A promoter responses to NRF1 overexpression in mammalian cells, reporter activity was studied in cells expressing intact NRF1, a deletion mutant analogous to EWGΔN144 (NRF1ΔN86), or the NRF1VP16 fusion. In HeLa cells, NRF1 expression produced ~3-fold activation of MEF2A-Luc and ~4-fold activation of MEF2Ap2-Luc (Fig. 4D). Although modest, these responses are equal to or greater than those reported for other promoter reporters in mammalian cells (7, 30). As expected, more robust effects were produced by NRF1VP16, which stimulated these reporters 12- and 15-fold.
respectively. These studies suggested that NRF1 co-regulates the MEF2A gene promoters through its proximal promoter 1 element.

**Myocyte mef2a mRNA Is Reduced with nrf1 RNA Interference**—We used RNAi to study the effects of nrf1 knockdown on expression of endogenous mef2a mRNA in muscle cells. C2C12 cells were transfected with dsRNA targeted to nrf1. Control cells received an siRNA that targets gapdh, one that fails to hybridize with any known message, or no dsRNA in a mock transfection. Transfection conditions were optimized using Alexa Fluor 488-tagged control siRNA. Because myoblasts proliferate rapidly, we performed serial transfections with a low concentration (5 nM) of the siRNAs. Total cell RNA, harvested at cell confluence after three transfections at doubling time intervals, was used in reverse transcriptase reactions. Validated primer pairs specific for gapdh and nrf1 were used in quantitative PCR, with values normalized to sample β-actin (actb) mRNA levels. Messenger RNAs of these targets were each selectively down-regulated to 8 and to 15% of control levels, respectively, with transfection of the cognate siRNA, as can be seen in stained electrophoretic gels of the reaction products (Fig. 5). Levels of the non-cognate siRNA-transfected cell and control cell mRNAs were not affected and were indistinguishable from those in mock-transfected cells.

Having established the conditions under which nrf1 expression in myocytes could be down-regulated, we tested the consequences for mef2a expression. As controls, we examined

![Figure 3](image-url)
effects on the expression of established targets of NRF1, including the 
cycs and cox5b genes that encode the somatic form of 
cytochrome c and the cytochrome c oxidase Vb subunit, respectively (4). Validated primer pairs for 
mef2a, cycs, and cox5b were 
used, with the former pair designed to detect all splicing vari-
ants of 
mef2a (18). In an experimental series, the respective 
mef2a, cycs, and cox5b mRNA expression levels were reduced in 
nrf1 siRNA-transfected cells to 33, 30, and 25% of control levels 
(Fig. 5). Together with the NRF1 overexpression study findings, 
this clearly demonstrates a strong correlation between cellular
NRF1 levels and MEF2A gene expression.

FIGURE 4. Forced expression of NRF1 stimulates 
MEF2A promoter activity. A, schematic of MEF2A promoter-reporters (19).
B, diagram of the domain 
structures of NRF1 and EWG, and mutants and fusions used. 
Drosophila S2 cells in 6-well plates (C) and HeLa cells in 12-well plates (D) were transfected in triplicate for each condition with indicated reporters (S2, 3 µg/well; HeLa, 1 µg/well) and SV40-gal (0.3 µg/well). After 48 h, cell extract luciferase activities were determined, and values were normalized for transfection efficiency using β-galactosidase. Data represent the average ± S.E. of 3 independent transfections.

FIGURE 5. nrf1 RNA interference leads to down-regulation of mef2a mRNA in cultured myocytes. Subconfluent C2C12 cells were transfected with 5 nM ds-siRNA directed at no gene (cntl, control), gapdh, or nrf1 at daily 
intervals for 3 days followed by RNA harvesting at cell confluence. QPCR was 
used on cDNA produced from these samples to determine nrf1, gapdh, mef2a, 
cycs, and cox5b mRNA levels in samples, each normalized to the respective 
actb level. One set of parallel reactions in a confirmatory QPCR was stopped at the midpoint of exponential amplification of the control condition sample, and reaction products were electrophoresed on 4% agarose gels containing ethidium bromide. Similar results were obtained in three independent siRNA 
transfections. 

MEF2A Promoter Activity Is Sensitive to NRF1 Element 
Mutation and to RNAi-mediated Down-regulation of nrf1 Expression—C2C12 transfections with a deletion series of both 
MEF2A promoter-reporters, including short (S), intermediate 
(I), and long (L) promoter constructs, showed that preferential activity in myocytes was maintained in the (S) reporters (19). These constructs each retain nearly 50% of full-length promoter function (Fig. 6, A and B). MEF2A reporters with an 
NRF1 element mutation corresponding to one that failed in binding competition assays (p1- and p2-[m1NRF1]-Luc) had 
markedly compromised activity compared with the cognate wild-type promoter reporters, consistent with positive control 
of MEF2A transcription from this element. Similar results were 
obtained in HEK 293 cells (not shown). This element therefore 
appears to be crucial to full promoter activity in a variety of cell 
types in which MEF2A is expressed.

To confirm that RNAi-mediated NRF1 underexpression 
reduced mef2a mRNA by a transcriptional mechanism, we 
evaluated MEF2A promoter-reporter activity in C2C12 and 
HEK 293 cells. In each case, cells were transfected with control 
or NRF1 siRNAs as for the mRNA studies followed after 48 h by co-transfection of the S-MEF2AP2-Luc reporter. NRF1 knock-
down led to significant reductions in MEF2A promoter activity to 42 and 25% of controls in C2C12 and 293 cells, respectively 
(Fig. 6C). The rapid proliferation rate of these cells probably 
means that this understates the impact of NRF1 down-regula-
tion on MEF2A promoter activity. Taken together with the 
NRF1 element mutation studies, these results confirm that 
NRF1 plays a critical role in MEF2A transcription in two diverse 
cell types, including differentiating muscle cells.

PGC1α Co-activates the MEF2A Promoters from the NRF1 
and MEF2 Elements—The close proximity of the MEF2A pro-
moter NRF1 and MEF2 elements suggested the possibility of an 
interaction. Indeed, the activities of reporters with or without 
mutations in the MEF2 and NRF1 sites were consistent with 
element cooperation (Fig. 7B). Under conditions where activity
of MEF2Ap2-Luc was ~5-fold higher than that of p2[m1NRF1]-Luc and 3-fold higher than a reporter with a MEF2 site mutation (p2[m1MEF2]-Luc) (19), it was nearly 20-fold higher than a reporter with mutations in both elements. Forced expression of MEF2A α2/β and NRF1ANB6 synergistically activated the MEF2A promoter in HeLa cells, also suggesting cooperation of the elements (Fig. 7A). No direct protein–protein interaction between MEF2 and NRF1 was noted in a mammalian two-hybrid system (not shown), eliminating one possible mechanism for element synergy.

NRF1 and MEF2 are each known to interact with PPARγ coactivator 1α (PGC1α) (32–36). This suggested that MEF2A transcription might be sensitive to the abundance of this cofactor. We therefore examined the sensitivity of MEF2A promoter activity to forced expression of this coactivator. As shown in Fig. 7B, PGC1α stimulated MEF2Ap2-Luc activity ~4-fold in C2C12 cells, which is similar to PGC1α activities on other promoters such as those of the TFBM genes (7). This effect is mediated from the NRF1 and MEF2 elements, as mutation of either site resulted in attenuation in the PGC1α effect, and the response was nearly abolished on p2[m1NRF1/m1MEF2]-Luc.

Co-recruitment of PGC1α is one possible mechanism for cooperative control of MEF2A transcription by MEF2 and NRF1.

Endogenous Muscle Cell MEF2A Binds the Promoters of Genes Encoding COX VIaH, PGC1α, and MEF2A—There are four MEF2 isotype genes in all mammals. These genes have distinct but overlapping expression patterns and functions (18, 26, 27). These genes encode either one protein (MEF2B) or multiple splicing variant proteins (MEF2A, MEF2C, and MEF2D) that have a common N-terminal DNA-binding and dimerization domain (17, 18), such that all MEF2 proteins can heterodimerize promiscuously. Expression of the MEF2 isoforms is induced at different stages of cultured myoblast differentiation, but each is highly expressed in differentiated myotubes (18). We therefore used C2C12 myotubes as substrate for ChIP assays to confirm occupation of gene promoters relevant to our hypothesis by MEF2A.

We developed a highly specific anti-MEF2A antibody that recognizes all splicing variants of MEF2A but no other MEF2 form. This antibody had been used by us previously to demonstrate MEF2A binding to the MEF2 element in the MEF2A promoter (19). We performed ChIP assays with C2C12 myotubes to verify that MEF2A proteins also bind the cox6αH and ppargc1α gene promoters at the respective MEF2 element regions. The gapdh gene promoter, which is not regulated or
FIGURE 9. MEF2A, with NRF1 and PGC1α, is part of a triad of transcriptional regulators of striated muscle metabolism. Key transcriptional pathways and representative targets of processes controlled by NRF1, MEF2A, and PGC1α are shown, including ramifications of MEF2A regulation by these three factors. Bold arrows indicate transcriptional control demonstrated in this or a recent publication (19). ΔALAS, δ-aminolevulinic acid synthase; MRP, mitochondrial RNA processing RNA; TOM20, translocase of outer membrane component 20.

bound by MEF2 proteins, served as a negative control. As shown in Fig. 8, MEF2A co-precipitated with chromatin containing the cox6ah and ppargc1a gene fragments but failed to precipitate gapdh. Thus, in this setting of co-expressed MEF2 isotype and splicing variant proteins, MEF2A forms occupy the promoters of MEF2A, COXH, and PPARGC1A. This work does not exclude co-occupation by other MEF2 forms.

DISCUSSION

Mito density is particularly rich in cardiac and skeletal muscle, where OXPHOS is brisk. The ETC is unique in these tissues by virtue of the expression of tissue-specific complex IV subunits, COX VIaH and COX VIIaH. Although the functions of these and other non-catalytic COX subunits are incompletely understood, there is evidence to suggest that COX VIaH confers sensitivity of COX to the cellular energy state. In specific, activity of COX isolated from striated muscle is activated by ADP, by virtue of the expression of tissue-specific complex IV subunits, COX VIaH and COX VIIaH. Although the functions of these and other non-catalytic COX subunits are incompletely understood, there is evidence to suggest that COX VIaH confers sensitivity of COX to the cellular energy state. In specific, activity of COX isolated from striated muscle is activated by ADP, whereas enzyme purified from other tissues is not (14). In addition, an antibody that specifically recognizes COX VIaH neutralizes this ADP sensitivity. It is therefore speculated that COX VIaH provides for rapid adaptation of ETC activity to energy stores in muscle, where energy demands can vary acutely and dramatically (1, 14). We explored a mechanism that could orchestrate tissue-specific expression of COX VIaH subunits at levels commensurate with other ETC components and the mito and mtDNA density in muscle. We found that COX VIaH gene promoters have evolutionarily conserved MEF2 elements, that myocyte MEF2A occupies the cox6ah promoter, and that MEF2A expression is in turn regulated by NRF1. We therefore propose that a NRF1 → MEF2A → COX VIaH cascade functions in parallel with the NRF → TFAM → ETCmito and NRF → ETCnucl pathways to control respiration in striated muscle (Fig. 9).

We have provided multiple lines of evidence that the closely approximated alternative MEF2A promoters are co-regulated by NRF1, the first limb of the proposed transcriptional cascade. We confirmed the evolutionary conservation of a canonical NRF1 binding site in MEF2A promoter 1. Other mobility shift assays with muscle cell nuclear extracts and myotube ChIP assays verified endogenous nuclear NRF1 protein association with this promoter. NRF1 homodimers recognize a 12-bp site, YGCGCAYGCGR, and there is little tolerance for sequence degeneracy (4). By consequence of this and the presence of multiple CpG doublets, canonical NRF1 sites are predicted to appear in a mammalian genome at a frequency of only 10⁻⁸ bp. This location at ~47 relative to the promoter 1 TSS (and ~600 bp upstream of the promoter 2 TSS) and our DNA binding study results strongly support a role for this site in regulating MEF2A transcription. We saw no change in NRF1 binding to the site during myoblast differentiation using either assay. This was not surprising because this factor is constitutively bound to its DNA targets (37, 38).

A second line of evidence in support of MEF2A as a target of NRF1 was provided by experiments using forced expression of NRF1, N-terminal deletion mutants of NRF1 and its Drosophila ortholog EWG, or a NRF1VP16 fusion protein. We initially showed that the MEF2A mRNA level in cultured HEK cells was strongly induced with expression of NRF1VP16. We used the VP16 transactivation domain fusion in these exploratory studies because the forced expression of native NRF1 is known to induce rather small changes in target gene expression. For example, NRF1 overexpression led to only an ~2-fold increases in TFAM promoter activity (32), and a synthetic minimal promoter containing four NRF1 elements is typically used to demonstrate transactivity (30, 32). Likewise, transgenic expression of NRF1 in muscle to a level that exceeded the endogenous level by a factor of 10 produced only 1.5- and 2-fold increases in cytochrome c (cytc) and δ-aminolevulinic acid synthase (δ-alas) mRNAs, respectively (28). NRF1VP16 was therefore a valuable tool in the detection of MEF2A as a potential NRF1 target. Unlike the case in cultured mammalian cells, exogenous expression of NRF1 or EWG has been shown by other investigators to produce potent activation of target gene promoters in Drosophila cells (20, 30). We were able to exploit this model system to develop further evidence for the authenticity of MEF2A as a target of NRF1. We also saw modest but significant effects of NRF1 overexpression on MEF2A promoter-reporters in mammalian cells that were similar to those reported for other promoters.

Evidence that specifically addressed the role of NRF1 in controlling MEF2A transcription in muscle came from studies of the function of MEF2A promoter-reporters in C2C12 myocytes. Mutation of the NRF1 element produced a drastic decrease in reporter activity. Furthermore, nrf1 RNAi in this and other cell types led to coincident reductions in nrf1 and mef2a mRNA levels, as well as diminished MEF2A promoter activity. We believe that the composite of evidence reported here confirms that MEF2A transcription is controlled by NRF1. MEF2A is expressed in neurons, adipocytes, in various immune
NRF1 Regulates MEF2A Expression

cell types, and in smooth muscle, in addition to skeletal and cardiac myocytes. We have previously provided evidence that MEF2A has only two promoters, p1 and p2, which do not have tissue-specific activities (19). Because NRF1 is ubiquitously expressed (39) and we have shown here that p1 and p2 are co-regulated by this factor, NRF1 can probably control MEF2A expression in non-muscle cells as well as in skeletal muscle and heart. The effect of NRF1 RNAi on MEF2A expression and promoter activity in HEK 293 cells supports this contention.

In differentiating mouse myoblasts in vitro, there is a quantum burst in mef2a expression at the time of cell cycle withdrawal (18). There is recent evidence that the transactivity of NRF1 is sensitive to cell cycle-regulated phosphorylation (40). In specific, Cdk4 or Cdk6, in association with the regulatory subunit cyclin D1, can phosphorylate NRF1 Ser-47, which leads to diminished transactivity. Disinhibition then occurs with cell cycle withdrawal because of the associated reduction in cyclin D1 levels and NRF1 DD. Negative regulation of NRF1 activity by this mechanism is consistent with previous studies of the EWG_{AN14} N-terminal deletion mutant (20), as well as with our present findings with this and the analogous NRF1_{AN100} mutant. In each case, the mutants were more potent transactivators of NRF1-responsive promoters than the full-length native factors.

In proliferating cells, we speculate that cyclin D1/Cdk4 activity on NRF1 may account for or be involved in the upsurge in mef2a expression noted at cell cycle withdrawal. This stated, NRF1 transactivity and DNA binding are also positively regulated by incompletely characterized serum-responsive phosphorylation events (30, 38) and possibly in response to cellular redox state (41). In addition, this factor may repress transcription of some or all target genes under some circumstances (37). This suggests additional regulatory complexity with relevance to MEF2A transcription that will require further study.

mef1 mRNA is detectable very early in embryonic development and is ubiquitously expressed in developing and mature tissues (11, 39). Disruption of murine mef1 leads to embryonic failure at a very early stage (3.5–6.5 days post-coitus) (11), prior to the appearance of mef2a during normal development. Tests of the role of NRF1 in controlling temporal and spatial expression of MEF2A in vivo will therefore require regulation interference with mef1 expression and/or conditional mef1 knockout, complemented by paired transgenic MEF2A and MEF2A[mef1] promoter studies. In addition to the NRF1 element, the MEF2A 5′ regulatory region has other conserved elements (supplemental Fig. S1). A canonical MEF2 element overlies the major TSS of one of the alternative TATA-less promoters, and MEF2 activity at this site confers transcriptional autoregulation and sensitivity of MEF2A expression to stress signaling (19). Additional sites of potential relevance to both muscle-specific expression and MEF2 function are also present, including E boxes and putative NRF2 sites. Explorations of the functions of each of these sites are progressing in our laboratory.

Despite evidence presented here that NRF1 controls MEF2A expression as part of a transcriptional cascade, we cannot exclude a role for other MEF2 genes in this network. Indeed, one of the several MEF2C promoters does have conserved NRF1 elements (supplemental Fig. S2), suggesting that this iso-type may also be a target. MEF2C proteins may therefore participate in the proposed transcriptional cascade in some cell types or circumstances. However, neither MEF2C, MEF2B, nor MEF2D mRNA was induced with forced expression of NRF1_{VP16} under conditions where cultured cell MEF2A expression was strongly stimulated. MEF2A may therefore play a unique role among the isoforms as intermediary in communicating NRF1 activity. This is compatible with the mefa−/− mouse phenotype. These animals have deficient and disorganized myocardial mito and a neonatal sudden death syndrome, consistent with a defect in oxidative metabolism (42).

Although it is clear that COX_{II} expression is controlled from promoter MEF2A elements, any of the four mammalian MEF2 isotope proteins could conceivably act at these sites. We had previously found that available anti-MEF2 antibodies do not display MEF2 isotope specificities (19). We used a new validated isotype-specific antibody and myoglobin chromat to demonstrate that endogenous MEF2A occupies the cox6ah promoter. Because disruption or knockdown of one MEF2 gene results in the dysregulation of other isotypes (42, 43), we believe that this is the best evidence for a direct regulation of COX_{II} genes by MEF2A, the second limb of our hypothesized transcriptional cascade. mef2a is expressed in striated muscle precursors in the developing embryo after day 9 post-coitus, and expression is maintained in differentiated tissues after birth (26, 42, 44, 45). In differentiating mouse myoblasts in vitro, mef2a expression increases dramatically upon cell cycle withdrawal (18), coincident with the appearance of cox6a2 (22). Thus, MEF2A abundance in muscle in vivo and in vitro closely mimics expression of the heart/muscle forms of COX Vla and Vlla. This contrasts with the expression pattern of mef2c, which is present at high levels during embryonic development but subsequently diminishes drastically (26, 44, 45), and mef2d, which is an early marker of the myogenic lineage and is maintained in differentiated skeletal muscle (44–46). This stated, the MEF2A N-terminal MAD5/MEF2 region that mediates dimerization and sequence-specific DNA binding is shared among all MEF2 proteins (26). We therefore cannot exclude co-regulation of the COX_{II} genes by other MEF2 forms or by heterodimers of MEF2A with MEF2B, MEF2C, or MEF2D at different stages of muscle development or differentiation.

MEF2A is highly expressed in skeletal muscle and heart, but it is also present at other sites including neural, adipose, and immune cells (17, 18, 26). Selective expression of COX_{II} genes may therefore require muscle-specific activities of either MEF2A or its collaborating factors. Certain splicing variants of MEF2A are expressed only in heart and muscle (27), providing one potential mechanism for a muscle-specific MEF2A activity. Signaling that controls MEF2 protein or cofactor modifications may also be operative, particularly among pathways regulated by intramyocellular Ca^{2+} transients (47). Myogenic basic helix-loop-helix factors could also contribute to muscle-specific expression, because these factors can use DNA-bound MEF2 as scaffolding to transactivate MEF2 target genes (48), and these factors could also regulate MEF2A transcription from

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7 T. Gulick, unpublished observations.
the promoter E boxes. The strict specificity of COX6AH expression suggests that gene silencing must also occur in non-muscle cells, the mechanisms for which remain to be determined.

The control of MEF2A transcription by NRF1 has implications beyond the coordinated co-expression of ETC subunits in muscle. Specifically, other genes that are regulated by MEF2A are implicated by our findings as potential indirect targets of NRF1. The GLUT4 gene provides one important example that has particular relevance to muscle and cardiac metabolism. Glut4 is the insulin-responsive facilitated glucose transporter form that provides the major route for glucose uptake in striated muscle (49–51). Both cultured myocyte and transgenic promoter-reporter studies have established that MEF2A regulates glut4 expression through a promoter MEF2F element (49, 50). Muscle tissue Glut4-level, insulin-stimulated glucose transport and MEF2A protein concentration were each found to be increased 2-fold in mice carrying a transgene that over-expresses NRF1 in skeletal muscle compared with control tissue (28). Our detection of MEF2A as a target of NRF1 suggests the relevant mechanism, NRF1 → mef2a → glut4, because glut4 is not a direct target of NRF1. Given the tissue expression patterns of both NRF1 and MEF2A, many more genes are likely to be regulated by such a cascade in both muscle and non-muscle sites.

PGC1α was originally identified as a coactivator of PPARγ and NRF1 in brown adipose tissue, but it is now recognized as a key regulator of mito biogenesis in various tissues (32, 52). PGC1α also coactivates MEF2 factors (33, 35, 36), and its forced expression in muscle increases mito density and OXPHOS and produces a fast-twitch/glycolytic to slow-twitch/oxidative phenotype conversion (53). It is established that PGC1α co-activates transcription from a PPARGC1A promoter MEF2 site (33, 35). We have shown here with myotube ChIP that MEF2A factors bind this PPARGC1A element where they are poised to recruit PGC1α in this autoregulatory loop. We have also shown that PGC1α co-activates the MEF2A promoter from its MEF2 and NRF1 elements, confirming that PGC1α can also feed back on MEF2A transcription. Taken together, this indicates that MEF2A, NRF1, and PPARGC1A and their respective protein products form a mutually reinforcing network of auto- and cross-regulation capable of directing mito biogenesis and OXPHOS capacity in muscle (Fig. 9). The NRF1 promoter has not been characterized, although the 5′-exons of human NRF1 have been identified (54). There is a canonical MEF2 binding site upstream of the human NRF1 TSS7 suggesting the possibility of direct reciprocal MEF2A and NRF1 regulation. We are exploring whether this vector is also part of this transcriptional network.

The regulation of MEF2A transcription by NRF1 and PGC1α is highly relevant to metabolic dysregulation in diabetes. Muscle glut4 mRNA, Glut4 protein, and insulin-stimulated glucose uptake are reduced in animal models of diabetes, and there is a coincident down-regulation of mef2a mRNA and MEF2A protein abundance (50, 51, 55). In these models, a hypercatabolic state leads to a decline in the [AMP]/[ATP] ratio and a coincident reduction in 5′AMP-activated protein kinase (AMPK) activity (55–58). Because expression of both mef2a and of glut4 can be restored with the administration of a small molecular activator of AMPK (55), this pathway is implicated as a crucial sensor linking cell energy state with the capacity for nutrient uptake and metabolism (55, 56). There is recent evidence to suggest that AMPK activity regulates nuclear DNA binding activity of both NRF1 (59) and MEF2F (60). Neither factor appears to be a direct target of AMPK (60). AMPK may therefore target co-repressor(s) of one or both factors to promote dissociation and de-repression of targets such as MEF2A (19, 61, 62). As one alternative, AMPK activity could indirectly influence the subnuclear locus or co-activator associations of these factors. In any case, down-regulated MEF2A expression may be a primary mechanism by which NRF1 and PGC1α targets are coordinately down-regulated in humans with diabetes and insulin resistance (63).

We saw no evidence for a direct protein-protein interaction between NRF1 and MEF2A. These factors may cooperate in the recruitment of transcriptional co-regulator(s) or the induction of chromatin remodeling to account for the observed functional synergy. Paired MEF2 and NRF1 elements also exist in other MEF2 gene regulatory regions, including the aforementioned MEF2C promoter (supplemental Fig. S2) and the Drosophila DMef2 II-E enhancer (supplemental Fig. S3). The DMef2 5′-region has various enhancers that govern the complex developmental and spatial expression of the sole MEF2 gene in the fly (64). II-E is responsible for transcriptional autoregulation (65) and for DMef2 expression near and after terminal differentiation of somatic muscle (64). We find that a canonical EWG element in this region binds NRF1 and governs enhancer activity (supplemental Fig. S3). Control of MEF2A transcription by NRF1 may therefore be conserved among higher metazoans. The sea urchin NRF1 ortholog, P3A2, directs territory-specific transcription of muscle genes during embryonic development (66), and EWG is known to regulate flight muscle development (31). Our work suggests a potentially relevant mechanism and establishes a foundation for in vivo functional analyses in various model systems to elucidate the contributions of NRF1/EWG to the developmental and spatial expression of MEF2 genes.

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