NRF-1: a *trans*-activator of nuclear-encoded respiratory genes in animal cells

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The assembly of the respiratory apparatus requires the coordinate expression of a large number of genes from both nuclear and mitochondrial genetic systems. In vertebrate organisms, the molecular mechanisms integrating the activities of these distinct genomic compartments in response to tissue demands for respiratory energy remain unknown. A potential inroad to this problem came with the discovery of nuclear respiratory factor 1 (NRF-1), a novel transcriptional activator defined by mutational and DNA binding analysis of the somatic cytochrome *c* promoter. Functional NRF-1 sites are now observed in several other recently isolated nuclear genes whose products function in the mitochondria. Among these are genes encoding subunits of the cytochrome *c* oxidase (subunit VIc) and reductase (ubiquinone-binding protein) complexes. In addition, a functional NRF-1 site resides in the MRP RNA gene encoding the RNA moiety of a ribonucleoprotein endonuclease involved in mitochondrial DNA replication. Synthetic oligomers of these sites competitively displace NRF-1 binding to the cytochrome *c* promoter. NRF-1-binding activities for each site also have the same thermal lability, copurify chromatographically, and make similar guanosine nucleotide contacts within each recognition sequence. Moreover, NRF-1 recognition *in vitro* correlates with the ability of each site to stimulate expression *in vivo* from a truncated cytochrome *c* promoter. The presence of NRF-1-binding sites in nuclear genes encoding structural components of the mammalian electron transport chain, as well as the mitochondrial DNA replication machinery, suggests a mechanism for coordination of nuclear and mitochondrial genetic systems through the concerted modulation of nuclear genes.

[**Key Words:** Electron transport chain; gene regulation; cytochrome *c*; cytochrome *c* oxidase; ubiquinone-binding protein; MRP RNA gene]

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The majority of gene products that specify the mitochondrial respiratory complexes in animal cells are encoded in the nuclear genome. The mitochondrial genome contributes only 13 respiratory subunits, along with rRNAs and tRNAs of the mitochondrial translational system (for review, see Attardi 1985). Therefore, in addition to specifying most of the structural and catalytic components directly involved in energy metabolism, nuclear genes must also control mitochondrial transcription, translation, and DNA replication. Despite the predominant role of the nuclear genome in specifying respiratory function, little is known about the regulation of nuclear-encoded respiratory genes in multicellular organisms.

The capacity for oxidative metabolism is a fundamental property of the differentiated state that varies widely among animal cells and tissues (Stotz 1939). These differences are often reflected in the number and size of mitochondria, as well as in the expression of respiratory gene products (Tata et al. 1963; Scarpulla and Wu 1983; Horrum et al. 1985; Scarpulla et al. 1986;

Robin and Wong 1988; Schwerzmann et al. 1989). A fundamental issue is how the biosynthetic activities of nuclear and mitochondrial genetic systems are coordinated to meet cellular energy demands. One mechanism would be to regulate the expression of nuclear genes required for mitochondrial transcription, translation, or replication in concert with those encoding respiratory subunits. For example, variation in mitochondrial DNA copy number may provide an important mechanism for controlling the level of mitochondrial gene expression (Williams 1986). Those nuclear gene products required for replication may therefore be focal points for coordinate regulation. Similarly, nuclear genes encoding the mitochondrial transcriptional or translational machinery may be coregulated with nuclear genes encoding respiratory subunits.

To begin to identify transcriptional activators specifically involved in coordinating the expression of nuclear respiratory genes in animal cells, the human and rat cytochrome *c* genes were isolated (Scarpulla 1984; Evans and Scarpulla 1988b; Virbasius and Scarpulla 1988) and an analysis of the rat somatic cytochrome *c* (RC4) promoter was performed (Evans and Scarpulla 1988a, 1989). One class of promoter sequences identified by mutational mapping and DNase I footprinting is recognized

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by nuclear factors common to many nonrespiratory genes. These include functional recognition sites for activating transcription factor (ATF) (Lee et al. 1987; Lin and Green 1988) in the upstream promoter and for Sp1 (Briggs et al. 1986) within the first intron (Evans and Scarpulla 1989). In addition to these, a novel nuclear factor designated nuclear respiratory factor 1 (NRF-1) was found to bind to a specific sequence in the upstream promoter. Binding of NRF-1 correlated with promoter stimulation, and a synthetic oligomer of the NRF-1 recognition site, when cloned in *cis*, stimulated the activity of a truncated cytochrome *c* promoter.

Recently, several other mammalian nuclear genes whose products either function directly in the respiratory chain or participate in mitochondrial genomic expression have been isolated. Two of these, the human cytochrome *c*₁ gene (hCC₁) (Suzuki et al. 1989a) and the human ubiquinone-binding protein gene (hQP) (Suzuki et al. 1989b), encode subunits of respiratory complex III (cytochrome *c* reductase), whereas a third, the rat cytochrome *c* oxidase subunit VIc gene (COX-VIc-2) (Suske et al. 1988), encodes a subunit of respiratory complex IV (cytochrome *c* oxidase). In addition to these genes for respiratory subunits, the genes for the mouse (Chang and Clayton 1989) and human (Topper and Clayton 1990) mitochondrial RNA processing (MRP) RNA have been isolated. These encode the RNA moiety of a ribonucleoprotein endonuclease involved in generating primer RNAs for mitochondrial DNA replication (Chang and Clayton 1987). They therefore represent a particularly interesting class of nuclear genes that function in mitochondrial genomic expression. In this study, we establish that functional NRF-1 recognition sites are present in nearly all of these nuclear genes. The sites from each gene are transcriptionally active and form indistinguishable DNA-protein complexes with NRF-1. By regulating both classes of genes, NRF-1 may help coordinate the expression of nuclear and mitochondrial genetic systems in response to cellular energy demands.

Results

NRF-1 recognition sites are common to nuclear genes whose products function in the mitochondria

The NRF-1 recognition site was originally defined in the RC4 gene as a 19-nucleotide GC-rich sequence element located between -167 and -149, corresponding to a strong DNase I footprint obtained by using either COS-1 or HeLa cell nuclear extracts (Evans and Scarpulla 1989). Mutations in this site diminished RC4 promoter activity, and a synthetic oligomer of the sequence was able to stimulate a truncated cytochrome *c* promoter. The NRF-1 recognition site is highly conserved (with one mismatch) in the human cytochrome *c* promoter (Fig. 1). Examination of the upstream regions of several recently isolated nuclear genes whose products function in the mitochondria revealed similarities to sequences encompassing the cytochrome *c* NRF-1 sites (Fig. 1). These include the COX-VIc-2 gene at positions -39/-28 and +39/+28, the hCC₁ gene at -449/-438, the hQP gene

at -53/-67, and the mouse MRP (mMRP) and human MRP RNA genes at -308/-297 and -292/-281, respectively.

To determine whether any of these sites could bind NRF-1, synthetic oligonucleotides containing each sequence were cloned into plasmids and tested for their ability to compete for NRF-1 binding to an oligonucleotide containing the RC4 promoter site (Fig. 2). Crude nuclear extracts from both COS-1 and HeLa cells produced an NRF-1-DNA complex of identical size (lanes 1 and 12), which was unaffected when an excess of cloning vector was included in the binding reaction as a nonspecific competitor (Fig. 2, lanes 2 and 13). Similarly, the formation of the NRF-1 complex was not inhibited by an excess of plasmids containing either the RC4 ATF transcription factor-binding site (RC4 -281/-256, lanes 3 and 14), or RC4 promoter region I (RC4 -187/-161, lanes 11 and 22). The latter is an independent *cis*-acting promoter element for which a *trans*-acting factor has yet to be identified (Evans and Scarpulla 1988a, 1989). In contrast, NRF-1 complex formation was strongly inhibited when the binding reactions contained an excess of cloned rat (RC4 -172/-147, lanes 4 and 15) or human (HCS -171/-154, lanes 5 and 16) cytochrome *c* NRF-1 sequences. Likewise, an excess of cloned oligomers containing the COX-VIc-2 +20/+46 (lanes 7 and 18), the hQP -66/-41 (lanes 9 and 20), and the mMRP RNA -311/-292 (lanes 10 and 21) sequences all eliminated NRF-1 binding, suggesting that these sites are biologically active. Although COX-VIc-2 -44/-20 (lanes 6 and 17) and hCC₁ -454/-431 (lanes 8 and 19) resemble the RC4 NRF-1 site, both failed to compete for NRF-1 binding. Alignment of those sequences active in this competition assay suggested a palindromic consensus sequence (T/C)GCGCA(T/C)GCGC(A/G) for the NRF-1 recognition element (Fig. 1). The mMRP and human MRP RNA genes both contain perfect matches to this sequence, whereas the RC4, COX-VIc-2 (+39/+26), and hQP genes all contain sites with a single mismatch to the consensus.

NRF-1 sites can be distinguished from other cis-acting elements requiring an alternating GC motif

A notable feature of the NRF-1 recognition sequence is the alternating GC motif. In fact, as shown by competition footprinting of the RC4 promoter, an excess of poly[d(I-C)] can compete for NRF-1 binding to the RC4 promoter without affecting the ATF footprint observed with the same promoter fragment (Fig. 3, cf. lanes 3 and 4). The E2F transcriptional activator also has a GC motif as part of its recognition sequence, and its binding is also inhibited by an excess of poly[d(I-C)] (SivaRaman and Thimmapaya 1987), raising the possibility that E2F and NRF-1 are identical or related proteins. However, two synthetic oligomers containing E2F-binding sites from the adenovirus E1A promoter (SivaRaman and Thimmapaya 1987) did not compete for NRF-1 binding to the RC4 promoter when present at either 20- or 100-fold molar excess (lanes 7-10). Under the same conditions,

Gene	Abbreviated Name	Sequence
Rat somatic cytochrome c	RC4	-173 atgctag CcCGCATGCGCG cgcacctt -147
Human somatic cytochrome c	HCS	-176 atgctcg CcaGCATGCGCG cgcaggtc -150
Rat cytochrome c oxidase VIc-2	COX-VIc-2	-46 gtgacca actGgATGCGCG tttgtata -20
Rat cytochrome c oxidase VIc-2	COX-VIc-2	+46 ctagcag CaCGCATGCGCA ggagccga +20
Human cytochrome c_1	hCC ₁	-456 tcccgcgt gGtGCACGCGCt gcctgcgg -430
Human ubiquinone-binding protein	hQP	-46 ggccgct TGCGCagGCGCA gttctgac -75
Mouse MRP RNA	mMRP RNA	-315 gccgtag TGCGCACGCGCA ggagacca -289
Human MRP RNA	hMRP RNA	-299 gcccaca CGCGCACGCGCA cgcgagca -273
Human cytochrome c_1 UP		tcccgcgt gGCGCACGCGCA gcctgcgg
		$\begin{matrix} T & & A \\ C & GCGCA & C \\ & C & GCGC \\ & & G \end{matrix}$

Figure 1. Comparison of NRF-1-binding site similarities in several nuclear genes encoding mitochondrial functions. Sequences from the HCS gene (Evans and Scarpulla 1988b), the COX-VIc-2 gene (Suske et al. 1988), the hCC₁ gene (Suzuki et al. 1989a), the hQP gene (Suzuki et al. 1989b), and the mouse (Chang and Clayton 1989) and human (Topper and Clayton 1990) MRP RNA genes are aligned for maximum identity with the RC4 gene NRF-1-binding site. The position of the sequence is indicated relative to the defined transcription start sites for the HCS, COX-VIc-2, and MRP RNA genes. The position of the hQP gene sequence is indicated relative to the 5'-end of a cDNA, whereas the position of the hCC₁ sequence is in reference to the translation initiation codon. The NRF-1-binding site created in the hCC₁ UP oligonucleotide is also shown. Uppercase letters denote matches to the proposed NRF-1 consensus binding site indicated in the bottom line.

either RC4 -172/-147 (lanes 5 and 6) or COX-VIc-2 +20/+46 (lanes 11 and 12) oligonucleotides displayed identical specificities in eliminating the NRF-1 footprint. Thus, the recognition specificity of NRF-1 was clearly distinguished from that of E2F when authentic promoter sequences were used as competitors. Additionally, although the NRF-1 recognition sequence is similar to the SV40 enhancer SphI motif (Wildeman et al. 1986), an SV40 enhancer and early promoter fragment failed to compete for NRF-1 binding (not shown). These results implicate the importance of the repeating GC

motif for NRF-1 recognition but clearly distinguish NRF-1 from other known activators whose binding sites contain similarities to this motif.

NRF-1 complexes formed with various respiratory promoter sites are indistinguishable

Several examples now exist where very closely related or identical promoter sequences are recognized by different nuclear transcription factors. For example, two distinct proteins, Oct-1 and Oct-2, bind to the octamer sequence

Figure 2. Competition of NRF-1 sequences from various respiratory promoters for NRF-1 binding to the RC4 promoter site. End-labeled RC4 -172/-147 oligonucleotide was incubated with 25 μ g COS-1 (lanes 1-11) or HeLa cell (lanes 12-22) nuclear extract and assayed for the formation of the NRF-1 shifted complex by gel electrophoresis. Competition binding reactions contained a 100-fold molar excess of plasmid DNA with no insert [pGEM-7Zf(+)] or with cloned oligonucleotides containing the RC4 ATF-binding site [RC4 -281/-256], the RC4 promoter element I [RC4 -187/-161], or the NRF-1-binding site homologies from the RC4 gene [RC4 -172/-147], the HCS gene [HCS -171/-154], the COX-VIc-2 gene [COX-VIc-2 -44/-20, COX-VIc-2 +20/+46], the hCC₁ gene [hCC₁ -454/-431], the hQP gene [hQP -66/-41], and the mMRP RNA gene [mMRP RNA -311/-292]. [None] No competitor.

Figure 3. Specificity of oligonucleotide competition for NRF-1 binding to the RC4 promoter. Approximately 10 fmole of 3'-end-labeled fragment containing the RC4 promoter between -326 and -66 was incubated in the absence (lane 2) or presence (lanes 3-11) of 25 μ g COS-1 nuclear extract and digested with DNase I. Competitors included 1.25 μ g poly[d(I-C)] $\cdot$ poly[d(I-C)] and 20- and 100-fold molar excesses of oligonucleotides containing NRF-1-binding sites RC4 -172/-147 (lanes 5 and 6) and COX-Vlc-2 +20/+46 (lanes 11 and 12), or E2F-binding sites [E1A -296/-272, E1A -234/-217] from the adenovirus E1A gene (lanes 7-10). The positions of the NRF-1 and ATF DNase I footprints are marked by vertical bars. (G) G reaction of the labeled fragment.

element (ATGCAAAT), resulting in DNA-protein complexes of different mobility (LeBowitz et al. 1989; Scholer et al. 1989). Similarly, DNA-protein complexes formed with oligonucleotides containing the ATF consensus sequence (TGACGTCA) display a heterogeneous banding pattern suggestive of multiple forms of ATF (Andrisani et al. 1988; Hai et al. 1988). To determine whether each of the respiratory promoter sites yielded a similar NRF-1 complex, the oligonucleotides used previously as competitors were end-labeled, incubated with HeLa cell nuclear extract, and assayed by mobility shift. As expected, RC4 -281/-256, containing a perfect match to the ATF site consensus, yielded multiple specific complexes (Fig. 4, lanes 1 and 2) that were not competed by an excess of the RC4 NRF-1 oligomer (lane 3). RC4 -187/-161 and hCC₁ -454/-431 did not form stable complexes (lanes 13-15, 22-24), whereas COX-

Vlc-2 -44/-20 yielded a very faint band (detected only on prolonged exposure of the autoradiograph; data not shown) that migrated faster than the NRF-1 complex and that was not decreased by competition with the RC4 NRF-1 oligomer. In contrast, those sequences that competed for NRF-1 binding to the RC4 NRF-1 site (COX-Vlc-2 +20/+46, hQP -66/-41, and mMRP -311/-292) formed complexes with identical electrophoretic mobility to the RC4 NRF-1 complex (Fig. 4, lanes 4, 10, 16, and 19). The formation of each of these complexes was inhibited by an excess of the RC4 NRF-1 oligomer (lanes 6, 12, 18, and 21), but not by the RC4 ATF oligomer (lanes 5, 11, 17, and 20), confirming their specificity for NRF-1.

Thermal inactivation experiments provide a second line of evidence that NRF-1 recognition of each promoter occurs through the same molecular species (Fig. 5). When nuclear extracts were preincubated at various temperatures and then assayed for the formation of a shifted complex with RC4 -172/-147, COX-Vlc-2 +20/+46, hQP -66/-44, and mMRP -311/-292 (Fig. 5), the loss of DNA binding activity with increasing temperature was identical for each of these oligomers. Preincubation of the nuclear extracts at 55°C strongly diminished the formation of each NRF-1 complex, whereas a 60°C pretreatment completely abolished NRF-1 binding. In contrast, the major ATF complexes formed with RC4 -281/-256 were unaffected by temperatures up to 65°C, although some of the minor species were diminished at lower temperatures. Additionally, the NRF-1-binding activities obtained with RC4 -172/-147, COX-Vlc-2 +20/+46, hQP -66/-41, and mMRP -311/-292 copurified when crude nuclear extracts were fractionated on a heparin/agarose column (Fig. 6) under conditions where the multiple ATF complexes formed with RC4 -281/-256 were partially resolved from each other and from NRF-1 activity. Thus, by these criteria, the NRF-1 complex formed with each respiratory promoter site was indistinguishable.

If recognition of each site occurs through the same molecular species, it is likely that the pattern of nucleotide contacts would be similar for each promoter. Methylation interference was performed to identify major groove guanosine nucleotides involved in NRF-1 binding to RC4, COX-Vlc-2, hQP, and mMRP NRF-1 sequences. As shown in Figure 7, binding of NRF-1 revealed either complete or partial methylation interference of similar nucleotides within the core recognition sequence from each gene. A summary comparison of the interference patterns obtained (Fig. 8) shows two clusters of contact sites centered over each of the directly repeated TGC/GCA motifs that comprise the core recognition site. Each cluster consists of three nucleotides common to every site, with the exception of the G at -60 on the upper strand of the hQP sequence. In addition to the nucleotides within each cluster, the interference pattern for each gene has at least one nucleotide that is not common to all of the others. For example, both hQP and MRP RNA genes show a strong interference at nucleotides -54 and -307, respectively, on the upper strand of

Figure 4. Comparison of specific NRF-1 complexes formed with various respiratory promoter sites. Approximately 15 fmoles of end-labeled oligonucleotides (indicated above each series of lanes) was incubated with 25 μ g of HeLa cell nuclear extract and analyzed by mobility-shift assay. The binding reactions contained either no competitor oligonucleotide (None, lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25), 2 pmoles of unlabeled RC4 -281/-256 oligonucleotide (lanes 2, 5, 8, 11, 14, 17, 20, 23, and 26), or 2 pmoles of unlabeled RC4 -172/-147 oligonucleotide (lanes 3, 6, 9, 12, 15, 18, 21, 24, and 27), as labeled above each lane.

the first repeat that does not appear in either RC4 or COX-Vic-2 sites. Likewise, RC4 has a strong interference at -158 on the lower strand of the second repeat that does not appear in any of the other sites. In addition, the RC4 site has multiple contacts in the GC motif immediately 3' to the NRF-1 core sequence element. These partial interference sites may reflect the binding of an auxiliary protein adjacent to the RC4 NRF-1 site that facilitates the binding of NRF-1 without affecting the mobility of the complex. Despite these individual differences, the majority of major groove contacts revealed by methylation interference are similar for all of the promoter sequences, suggesting that a similar or identical NRF-1 molecule is responsible for recognition of each. Although the interference patterns are not completely symmetrical for any of the binding sites, their correspondence with the tandemly repeated GC motifs within the consensus sequence suggests that NRF-1 may bind DNA as a dimeric molecule.

NRF-1 recognition of respiratory genes in vitro correlates with promoter stimulation in vivo

To compare directly the ability of these sequences to

contribute to promoter function in vivo, NRF-1 oligomers were cloned upstream of a truncated RC4 promoter-CAT (chloramphenicol acetyltransferase) expression vector (RC4CATBΔ/-66BΔ). When transfected into COS-1 cells, using pGEM-4 blue as a nonspecific carrier DNA, RC4 -172/-147 stimulated promoter activity 7-fold, whereas COX-Vic-2 +20/+46, hQP -66/-41, and mMRP -311/-292 all stimulated activity ~20-fold (Table 1). In contrast, those sequences resembling the NRF-1 recognition site, but unable to bind NRF-1 (COX-Vic-2 -44/-20, hCC₁ -454/-431), elevated the level of CAT activity only weakly.

The effects of mutations in the NRF-1-binding site of the intact RC4 promoter are observed only upon transfection with low amounts of cytochrome *c* promoter expression vectors (Evans and Scarpulla 1989), suggesting that COS-1 cells contain limiting amounts of NRF-1 available for stimulation of transfected promoters. In addition, cotransfection of a large number of RC4 NRF-1-binding sites in *trans* abolishes promoter stimulation by the cloned RC4 -172/-147 oligonucleotide (Evans and Scarpulla 1989). Thus, if the in vivo stimulation of promoter activity by COX-Vic-2 +20/+46, hQP -66/-41, and mMRP -311/-292 required the binding of NRF-1,

Figure 5. Thermal inactivation of NRF-1 binding activity. HeLa cell nuclear extract was incubated for 10 min at the indicated temperatures. After brief centrifugation to remove denatured proteins, the supernatants were assayed for the formation of shifted complexes with the oligonucleotides RC4 -172/-147, COX-Vic-2 +20/+46, hQP -66/-41, mMRP RNA -311/-292, and RC4 -281/-256. (Lane 2) The binding reaction contained a 100-fold molar excess of unlabeled RC4 -172/-147 as a competitor, except for binding reactions with labeled RC4 -281/-256 oligonucleotide, where a 100-fold molar excess of unlabeled RC4 -281/-256 oligonucleotide was included as a competitor.

it should be possible to diminish their stimulatory effects by cotransfection of a large number of NRF-1-binding sites in *trans*. To introduce a high copy (300-fold molar excess) of NRF-1 sites in *trans*, plasmids containing four tandem copies of either RC4 -172/-147 (p4×RC4 -172/-147) or mMRP -311/-292 (p4×mMRP -311/-292) were used as carrier DNA in transfection experiments. Under these conditions, both plasmids substantially reduced the stimulation of CAT activity by RC4 -172/-147, COX-Vic-2 +20/+46, hQP -66/-41, and mMRP -311/-292. The mMRP -311/-292 sequence functioned as a stronger competitor than RC4 -172/-147, reflecting the relative strengths of the RC4 and mMRP sites when positioned in *cis*. The weak stimulation of hCC₁ -454/-431 was also eliminated by the presence of a large excess of NRF-1-binding sites in *trans*, perhaps reflecting weak NRF-1 binding to this site in vivo. In contrast, the weak stim-

Figure 6. Copurification of NRF-1 binding activities on heparin-agarose. End-labeled oligonucleotides were incubated with various extracts and subjected to mobility-shift analysis. (Lane A) Twenty-five micrograms of HeLa cell nuclear extract was included in the binding reaction; (lane B) 25 μ g HeLa cell nuclear extract plus a 100-fold molar excess of unlabeled RC4 -172/-147 oligonucleotide (for labeled RC4 -172/-147, COX-Vic-2 +20/+46, hQP -66/-41, and mMRP RNA -311/-292 oligonucleotides) or unlabeled RC4 -281/-256 (for labeled RC4 -281/-256 oligonucleotide) was included in the binding reaction. All other binding reactions contained 20 μ l of column fractions derived from heparin-agarose chromatography of HeLa cell nuclear extract. Crude HeLa cell nuclear extract was applied to the column, and the flowthrough was collected (lane C). After washing the column with buffer D containing 0.1 M KCl (lane D), the column was eluted with a linear gradient of KCl in buffer D (fractions 6-26).

ulation of CAT activity by COX-Vic-2 -44/-20 was not diminished by the presence of excess NRF-1-binding sites and may have resulted from stimulation by a factor other than NRF-1. The ability of an excess of both RC4 and mMRP NRF-1 sites to compete in vivo strongly indicates that the observed promoter stimulation depends on the specific interaction of NRF-1 with its recognition site from the various respiratory genes.

Although the hCC₁ site has a strong match to the NRF-1 consensus (9 of 12 nucleotides), it is inactive both in factor binding in vitro and in promoter stimulation in vivo. In contrast, the NRF-1 site from the hCC₁ gene

Figure 7. Methylation interference analysis of NRF-1 binding. End-labeled DNA fragments containing the cloned RC4 -172/-147, COX-VIc-2 +20/+46, hQP -66/-41, and mMRP RNA -311/-292 oligonucleotides were partially methylated and subjected to preparative scale mobility-shift analysis by using HeLa cell extract fraction HA45 (see Materials and methods). (F and B) The DNA fragment recovered from the free and bound bands, respectively. The position of the NRF-1 sequence element within each DNA fragment is indicated by vertical bars. (●) Guanosine bases that strongly inhibited NRF-1 binding when methylated; (○) guanosines where methylation partially interfered with NRF-1 binding.

Figure 8. Comparison of the methylation interference pattern of the RC4, COX-VIc-2, hQP, and mMRP NRF-1-binding sites. The sequence of each cloned oligonucleotide fragment between the flanking *Bam*HI and *Hind*III sites is shown, with the end points of each promoter sequence denoted and the position of the NRF-1 sequence element overlined. (●) Methylation of guanosines strongly interfered with NRF-1 binding; (○) methylation of guanosines partially interfered with NRF-1 binding. Triangles above and below the consensus sequence indicate methylated guanosines in the upper and lower strands, respectively, which inhibited NRF-1 binding to each of the individual binding sites.

with a 10 of 12 nucleotide match to the consensus binds in vitro (Fig. 2) and stimulates promoter activity in vivo (data not shown). If the same factor is responsible for both in vitro binding and in vivo promoter activities, a simple mutation of two nucleotides to increase the similarity of hCC₁ -454/-431 to the NRF-1 consensus should restore both activities. The hCC₁ UP mutation (hCC₁ -454/-431 UP) was constructed for this purpose (Fig. 1). As shown in Figure 4, lanes 25-27, the mutation of two nucleotides was sufficient to confer specific NRF-1-binding activity on the formerly inactive hCC₁ sequence. In addition, hCC₁ -454/-431 UP stimulated the truncated cytochrome *c* promoter as strongly as the intact RC4 NRF-1 site (Table 1). As with the other NRF-1 recognition sites, this enhanced activity was diminished by cotransfection with an excess of cloned RC4 or MRP NRF-1 sites in *trans*. These results further confirm the correlation between in vitro and in vivo activities and strongly suggest that the same NRF-1 factor is responsible for both.

NRF-1 sites function within the proper promoter context

Although the effects of NRF-1 site mutations have been defined within the context of the intact cytochrome *c* promoter (Evans and Scarpulla 1989), it was of interest to determine whether these sites also function in the context of other respiratory promoters in which they occur. For this purpose, a fragment from the COX-VIc-2 gene, containing the 5'-flanking DNA (to -173 from the transcription start) and a portion of the first intron

Table 1. Stimulation of promoter activity by cloned oligonucleotides

Cloned oligonucleotide ^a	Relative CAT activity ^b		
	pGEM-4 blue	p4 × RC4 –172/–147	p4 × mMRP RNA –311/–292
None	1.0	1.0	1.0
RC4 –172/–147	6.7	2.3	2.5
COX-Vlc-2 –44/–20	1.7	2.4	1.6
COX-Vlc-2 +20/+46	25.6	5.0	3.4
hCC ₁ –454/–431	1.9	0.7	0.6
hQP –66/–41	22.4	9.9	5.9
mMRP RNA –311/–292	19.6	7.3	4.2
hCC ₁ –454/–431 UP	6.1	0.7	0.8

^aThe indicated oligonucleotides were cloned upstream of the cytochrome *c* gene truncated at position –66 in the vector pRC4CATBΔ/–66bΔ, as described in Materials and methods.

^bPromoter vectors (0.5 μg) were transfected into COS-1 cells by using 20 μg either pGEM-4 blue, p4 × RC4 –172/–147, or p4 × mMRP –311/–292 as carrier DNA. CAT activities were normalized for transfection efficiency by Hirt DNA analysis. The activity produced by the parent promoter vector with no cloned oligonucleotide was defined as 1.0 for each carrier DNA.

within the 5'-untranslated region (to +404), was cloned into a comparable position (+308) within the cytochrome *c* first intron in the original cytochrome *c* expression vector (Fig. 9A). The COX-Vlc-2 NRF-1 site was disrupted by a *Bgl*II linker insertion, and the wild-type and mutant constructs were assayed for both promoter activity and NRF-1 footprinting. As shown in Figure 9, disruption of the COX-Vlc-2 NRF-1 site resulted in a reduction in promoter activity (Fig. 9A) and the complete elimination of the NRF-1 DNase I footprint (Fig. 9B). These results are comparable in magnitude to those obtained with similar mutations in the cytochrome *c* NRF-1 site (Evans and Scarpulla 1989) and confirm that the NRF-1 sites in both genes are functional within the context of their promoters.

Discussion

Rat and human cytochrome *c* genes were initially isolated to serve as a model system to investigate the regulation of nuclear genes encoding mitochondrial respiratory functions in animal cells (Scarpulla 1984; Evans and Scarpulla 1988b; Virbasius and Scarpulla 1988). Studies of the RC4 promoter have revealed multiple control regions both upstream and within the first intron (Evans and Scarpulla 1988a, 1989). Some of these regions are the targets for nuclear factors, such as ATF (Lin and Green 1988) and Sp1 (Briggs et al. 1986), which also activate many other unrelated genes. In general, the structure of the promoter reflects the dual nature of cytochrome *c* expression in that the gene provides a housekeeping function yet is also regulated hormonally according to tissue-specific demands for respiratory energy (Booth and Holloszy 1975; Scarpulla et al. 1986). For example, some features of the upstream region, such as its GC richness with no conserved TATA box, are common to many housekeeping promoters (Melton et al. 1986; Sehgal et al. 1988), whereas others such as conserved CCAAT homologies and an ATF recognition site are characteristic of regulated promoters. The most notable control region consists of two adjacent but functionally

independent *cis*-acting elements upstream from the CCAAT homologies which, together, have a potent effect on promoter function. One of these is a recognition site for a novel nuclear factor designated as NRF-1.

Recently, several other mammalian nuclear genes encoding gene products involved in mitochondrial respiration or genomic expression have been characterized (Suske et al. 1988; Chang and Clayton 1989; Suzuki et al. 1989a, 1989b; Topper and Clayton 1990), providing an opportunity to consider these along with the cytochrome *c* genes as a family of functionally related genes. A common feature of all of the genes is that they have strong sequence similarities to the cytochrome *c* NRF-1 recognition site in their upstream regions. The NRF-1 sequences in the human and mouse MRP RNA genes (Topper and Clayton 1990) are perfectly conserved in a region of otherwise weak sequence conservation, suggesting an important function.

In this study, a number of criteria were applied to establish that functional NRF-1 sites reside in all but the hCC₁ gene: (1) Synthetic oligomers of each site competed for NRF-1 binding to the cytochrome *c* promoter; (2) complexes of identical mobility were formed with the NRF-1 oligomer from each gene, and complex formation was specifically displaced by an excess of RC4 NRF-1 sites; (3) the NRF-1-binding activity to each promoter site had the same thermal lability and copurified upon heparin-agarose fractionation of extracts; (4) NRF-1 binding to each site resulted in similar guanosine nucleotide contacts within the major groove over one full turn of the DNA helix; (5) each of the NRF-1 sites having DNA binding activity in vitro stimulated the activity of a truncated cytochrome *c* promoter in transfected cells. Moreover, an excess of NRF-1-binding sites in *trans* diminished promoter activity, confirming a requirement for NRF-1 recognition of each site. A 2-nucleotide mutation enhancing the similarity of the inactive hCC₁ site to the consensus restored both in vitro binding and in vivo promoter activities, strongly indicating that the same factor is responsible for both. Finally, an insertional disruption of the COX-Vlc-2 NRF-1

Figure 9. Function of the NRF-1 site in the intact COX-Vlc-2 promoter. (A) The COX-Vlc-2 promoter expression vector contained the COX-Vlc-2 gene from -173 to $+404$ (*BstEII*) fused to the cytochrome *c*/CAT expression vector intron at position $+308$ [(line) the COX-Vlc-2 upstream promoter; (solid region) the 5'-untranslated region exon of COX-Vlc-2; (open region) the fused COX-Vlc-2/RC4 intron; (hatched region) the CAT-coding region]. The NRF-1-binding site was mutated by insertion of a *BglII* linker at the *SphI* site within the NRF-1-binding sequence. Each vector (50 ng) was transfected into COS cells by using 20 μ g of pGEM-4 blue as carrier DNA. The relative CAT activities were normalized for transfection efficiency by analysis of Hirt DNA and were based on six independent transfections. (B) Fragments containing the intact COX-Vlc-2 promoter (lanes 1–5) or the COX-Vlc-2 promoter with a *BglII* linker inserted at the *SphI* site (lanes 6–10) were 3'-end-labeled on the noncoding strand and subjected to DNase I footprint analysis by using no extract (lanes 2 and 7) or 25 μ g of HeLa cell extract fraction HA45 (lanes 3–5, 8–10). Binding reactions contained no competitor (lanes 2, 3, 7, 8) or 100-fold molar excesses of COX-Vlc-2 $+20/+46$ (lanes 4 and 9) or RC4 $-172/-147$ (lanes 5 and 10) oligonucleotides. The position of the NRF-1 sequence element is indicated by the vertical bar. (G) G reaction of the labeled fragments.

site diminished both promoter activity and the NRF-1 footprint at this site, confirming that the NRF-1 site is functional within the context of the COX-Vlc-2 promoter. Similarly, a deletion mutation removing a segment of the mouse MRP RNA gene containing the NRF-1 recognition sequence was recently found to reduce the expression of this gene (E.F. Michelotti and D.A. Clayton, pers. comm.). Thus, for three of these nuclear genes (RC4, COX-Vlc-2, and MRP RNA), the effects of NRF-1 were verified within the context of their promoters. Taken together, these results establish that NRF-1 is a common activator of many nuclear genes that specify respiratory function in animal cells.

The products of the known genes containing functional NRF-1 sites operate at several levels. The COX-Vlc-2 gene product is an integral membrane subunit of cytochrome *c* oxidase (complex IV), the terminal enzyme in the respiratory chain considered to be the rate-limiting step (for review, see Poyton et al. 1988). Cytochrome *c* is a dissociable heme protein that serves as an electron donor in the cytochrome oxidase reaction (for review, see Hatefi 1985). The hQP gene encodes the cytochrome *c* reductase (complex III) subunit responsible for binding ubiquinone (coenzyme Q). This lipid-soluble electron carrier couples the NADH (complex I) and succinate (complex II) dehydrogenase complexes to the remainder of the respiratory apparatus (for review, see Hatefi 1985). Thus, the potential effects of NRF-1 on respiratory chain expression are unlikely to be restricted to a single respiratory complex. A recently described patient with a severe respiratory chain defect affecting both complexes III and IV had diminished cytochromes *b*, *c* + *c*₁ and *aa*₃ in skeletal muscle mitochondria but little change in cytochrome *c*₁ polypeptide (Takamiya et al. 1986). On the basis of the results presented here, this phenotype might be predicted for a defect in NRF-1. It will be of considerable interest to determine whether genetic lesions affecting NRF-1 result in defective respiratory chain expression in patients with respiratory insufficiencies.

In addition to genes having important respiratory functions, the MRP RNA genes represent an interesting class of nuclear genes involved in mitochondrial genomic expression (Chang and Clayton 1989). The MRP endonuclease cleaves nascent light-strand transcripts at a conserved sequence within the mitochondrial D loop region, thereby generating RNA primers for heavy-strand DNA replication (Chang and Clayton 1987). In mammalian striated muscle, oxidative capacity varies with the number of mitochondrial genomes per cell and with the expression of mitochondrial gene products (Williams 1986). It may therefore be useful to coordinately regulate nuclear genes involved in replication with those contributing respiratory subunits as a means of communicating nuclear regulatory events to the mitochondria. The occurrence of NRF-1 sites in nuclear genes for both MRP RNA and respiratory proteins is suggestive of a model where NRF-1 helps coordinate nuclear and mitochondrial genomic expression through the concerted modulation of nuclear genes. One prediction

of this model is that nuclear genes encoding important components of the mitochondrial transcriptional or translational apparatus may also contain functional NRF-1 sites. A particularly attractive candidate would be the nuclear gene encoding mitochondrial transcription factor 1, which activates transcription by binding to both mitochondrial heavy- and light-strand divergent promoters (Fisher et al. 1987).

In yeast, transcriptional activators affecting cytochrome *c* expression have also been identified (for review, see Forsburg and Guarente 1989). The HAP2/3/4 complex is likely involved in the expression of several respiratory chain genes in yeast and appears to be equivalent to CP1, a mammalian CCAAT box transcription factor. A second activator, HAP1, mediates heme regulation by recognizing upstream activation sites that differ in sequence. However, HAP1 binding to each site includes nucleotide contacts over two helical turns on one side of the DNA helix in both the major and minor groove, with a single opposite major groove contact at one end of the binding site (Pfeifer et al. 1987). In contrast, the methylation interference patterns presented here suggest that NRF-1 recognition differs from HAP1 by making major groove contacts on both faces of the helix over a single helical turn. In addition, hemin stimulates the binding of HAP1 in vitro (Pfeifer et al. 1987) but does not appear to affect NRF-1 binding to the RC4 recognition site (not shown).

The discovery of functional NRF-1 sites in the majority of recently isolated mammalian respiratory genes raises interesting questions regarding the role of NRF-1 in mitochondrial biogenesis. First, is NRF-1 a constitutive activator required for basal level expression or does it mediate a transcriptional response to physiological signals? Thyroid hormones are the most well-known physiological effectors of respiratory metabolism in mammalian systems. Their effects on oxygen consumption, respiratory rate, the biosynthesis of respiratory proteins, overall mitochondrial biogenesis (Tata et al. 1963; Horrum et al. 1985), and the transcriptional induction of respiratory genes (Scarpulla et al. 1986) have been documented. NRF-1 may be an important component in a signal transduction pathway mediating thyroid hormone effects upon respiratory metabolism. Second, is NRF-1 control exclusive to nuclear respiratory genes? A computer search suggests that NRF-1 sites are not generally present in other housekeeping genes. However, if the major role of NRF-1 is in mitochondrial biogenesis, it seems likely, given the magnitude of the task of synthesizing and maintaining a major cellular organelle, that NRF-1 may operate on other genes involved in key biosynthetic activities. For example, the presence of MRP RNA in the nucleus is suggestive of a nuclear, as well as a mitochondrial, function (Chang and Clayton 1987; Yuan et al. 1989). Finally, are the nuclear genes for respiratory subunits independently regulated? The failure to detect a functional NRF-1 site in the hCC₁ gene suggests that not all nuclear respiratory genes are under NRF-1 control. Interestingly, cytochrome *c*₁ appears to be the only respiratory cytochrome whose biosynthesis is not

thyroid hormone responsive (Horrum et al. 1985). Similarly, NRF-1 sites appear to be absent in the rat testis-specific cytochrome *c* promoter region, suggesting that its expression is NRF-1 independent (Virbasius and Scarpulla 1988). An alternative explanation for these findings is that NRF-1, like yeast HAP1 and mammalian C/EBP, Oct-1, and Oct-2 factors (for review, see Johnson and McKnight 1989), recognizes sites with no obvious sequence similarity. Further characterization of NRF-1 and its cognate promoters should resolve these questions.

Materials and methods

Construction of plasmids and expression vectors

DNA sequences of oligonucleotides used in this study were as follows:

RC4 - 281/-256:	AATTCGCCGGAGGGTGACGTCATCCACGTCGG GCGGCTCCCACTGCAGTAGGTCAGGCCATG
RC4 - 172/-147:	GATCCTGTAGCCGCGATGCGCGCGCACCTTA GACGATCGGGCTACGCGCGGTGGAATTCGA
HCS - 171/-154:	GATCCGCCAGCATGCGCGCGCA GCGGTCGTACGCGCGGTTCTGA
COX-VIc-2 - 44/-20:	GATCCGACCAACTGGATGCGCGTTTGTATA GCTGGTTGACCTACGCGCAACATATTCTGA
COX-VIc-2 + 20/+46:	GATCCTAGCAGCAGCATGCGCAGGAGCCGA GATCGTCGTCGTACGCGTCTCGGCTTCGA
hCC ₁ - 454/-431:	GATCCGCTGGTGACGCGCTGCTCTGCGA GCGACCACTGCGCGACGCGCTTCGA
hCC ₁ - 454/-431 UP:	GATCCGCTGGCGACGCGCAGCTGCGA GCGACCGCTGCGCGTCTCGGACGCTTCGA
hQP - 66/-41:	GATCCAGAAAGCGCGCTGCGCAGGCGCAGTA GTCTTTCGGCGCAACGCGTCCGCTCATTCTGA
mMRP RNA - 311/-292:	GATCCTAGTGCACGCGCAGGAGA GATCACGCGTGGCGTCTCTCTTCGA
RC4 - 187/-161:	GATCCGGTACAACCTACCATGTAGCCCGCA GCCATGTTGGATGGTACGATCGGCGGTTCTGA

Single-stranded oligonucleotides were synthesized on an Applied Biosystems 4 DNA synthesizer, purified on Applied Biosystems oligonucleotide purification cartridges, and annealed to form double-stranded DNA oligonucleotides, as described previously (Kadonaga and Tjian 1986). The double-stranded oligonucleotides were cloned into the vector pGEM-7Zf(+), except for the RC4 - 281/-256 oligonucleotide, which was cloned into the vector pGEM-4 blue. Oligonucleotide sequences were verified by the dideoxy sequencing method (Sanger et al. 1977). The plasmids p4×RC4 - 172/-147 and p4×mMRP - 311/-292 were constructed by converting the *Hind*III site flanking the cloned oligonucleotide to a *Bgl*II site, followed by two rounds of dimerization by use of the flanking *Bam*HI and *Bgl*II sites, as described previously (Rosenfeld and Kelly 1986).

The vector pRC4CATBΔ/-66Δ was constructed by cloning a *Xho*I-*Kpn*I fragment containing a RC4-CAT fusion gene deleted of cytochrome *c* sequences upstream of position -66 (Evans and Scarpulla 1988a) into the vector pGEM-7Zf(+) at the *Sma*I (converted to a *Sal*I site) and *Kpn*I sites. Annealed oligonucleotides were cloned into the *Hind*III/*Bam*HI sites located in the pGEM-7Zf(+) polylinker, positioning the oligonucleotides 95 bp upstream of the cytochrome *c* transcription start site.

For analysis of the COX-VIc-2 promoter, a *Xho*I-*Pst*I fragment, containing the RC4-CAT fusion gene deleted of sequences upstream of -66, was inserted into the vector pGEM-4 blue at the *Kpn*I (converted to a *Xho*I site) and *Pst*I sites. In the plasmid pCOXVIcCAT/-173, a 396-bp *Eco*RI-*Bst*EII fragment

containing the cytochrome *c* promoter, first exon, and 5' half of the first intron was replaced with a 576-bp fragment from the rat COX-Vlc-2 gene containing the oxidase promoter (to -173), first exon, and 5' half of the first intron. The NRF-1-binding site in the COX-Vlc-2 gene was disrupted by digestion with *Sph*I, treatment with T4 DNA polymerase, and addition of a *Bgl*II linker.

Preparation and column chromatography of nuclear extracts

Nuclear extracts were prepared from either COS-1 cells grown in monolayer culture or HeLa cells grown in spinner culture by the method of Dignam et al. (1983). For thermal inactivation experiments, 150 μ g of HeLa cell crude nuclear extract in 30 μ l buffer D [20 mM HEPES (pH 7.9), 20% (vol/vol) glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.1 M KCl] was incubated for 10 min at the desired temperature, after which denatured protein was removed by centrifugation for 2 min at 4°C. Four microliters of each supernatant were tested for the formation of shifted complexes by mobility-shift assays.

For analytical heparin/agarose chromatography, 10 mg HeLa cell crude nuclear extract was applied to a column of heparin-agarose (0.7 $\times$ 6.0 cm) equilibrated in buffer D containing 0.1 M KCl at a flow rate of 15 ml/hr. The column was washed with 15 ml of the same buffer and eluted with a 50-ml linear gradient of buffer D containing 0.1–1.0 M KCl. Fractions (2.5 ml) were collected and analyzed in mobility-shift assays. Fraction HA45 was prepared by application of 125 mg of HeLa cell crude nuclear extract to a heparin-agarose column (2.5 $\times$ 6.0 cm). After washing the column with buffer D containing 0.1 M and 0.25 M KCl, NRF-1 was eluted with buffer D containing 0.45 M KCl. Ammonium sulfate was added to 50% saturation, and the precipitated protein was resuspended in buffer D, yielding fraction HA45.

Mobility-shift assays and DNase I footprinting

Annealed oligonucleotides were 3'-end-labeled by using Klenow enzyme and purified by gel electrophoresis. Binding reactions were performed in 25 μ l of TM buffer [25 mM Tris (pH 7.9), 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM KCl, 10% (vol/vol) glycerol] containing 4 μ g of sonicated calf thymus DNA, 25 μ g of nuclear extract, and ~15 fmoles of labeled oligonucleotide. For competition reactions, either 3 μ g (1.5 pmole) plasmid DNA or 1.5 pmole unlabeled oligonucleotides was added to the binding reaction prior to the addition of labeled oligonucleotide. After incubation at room temperature for 15 min, the samples were electrophoresed on a 5% polyacrylamide gel (acrylamide : bis, 58 : 1) in 45 mM Tris, 45 mM boric acid, and 1 mM EDTA (0.5 $\times$ TBE) at 10 V/cm for 2.5 hr. Gels were then dried and autoradiographed. Heparin-agarose column fractions were assayed similarly, except that 0.8 $\times$ buffer D was used in the binding reactions. DNase footprinting was performed as described previously (Evans and Scarpulla 1989).

Methylation interference

Derivatives of the vector pGEM-7Zf(+) containing the cloned oligonucleotides were linearized with either *Xho*I or *Mlu*I and 3'-end-labeled with Klenow enzyme. After a second digestion with either *Xho*I or *Mlu*I, the resulting fragments were gel-purified and electroeluted. Each fragment (1.0 $\times$ 10⁶ cpm, ~0.5 pmole) was treated with dimethylsulfate for 5 min at room temperature essentially as described (Maxam and Gilbert 1980). Binding reactions contained 5.0 $\times$ 10⁵ cpm methylated fragment and 80 μ g extract fraction HA45. Following electrophoresis, the wet gel was autoradiographed for 2 hr at 4°C, and the free and protein-bound DNA bands were located, excised, and

electroeluted. Gel fragments containing the protein-bound bands were soaked for 15 min in 0.5 $\times$ TBE, 0.2% (wt/vol) SDS prior to electroelution. The recovered fragments were extracted once with phenol/chloroform, ethanol-precipitated with 10 μ g *Escherichia coli* tRNA, cleaved at methylated guanines by treatment with 1 M piperidine, and electrophoresed on a 12% denaturing acrylamide gel.

Cell culture and transfections

COS-1 cells were grown and transfected by the CaPO₄ method as described (Evans and Scarpulla 1988a). Forty-eight hours following transfection, cells from triplicate plates were harvested into 3 ml of phosphate-buffered saline. One-half of these pooled cells was used for preparation of cell lysates for CAT assays, and one-half was used for the preparation of low-molecular-weight DNA by the Hirt (1967) method to normalize for transfection efficiency. Promoter activity values were the average of between two and five separate transfections of three plates each.

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