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Nuclear Control of Respiratory Chain Expression in Mammalian Cells

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The majority of gene products required for mitochondrial respiratory function are encoded in the nuclear genome. These include most of the respiratory subunits and all of the proteins that regulate the mitochondrial genetic system. One approach to understanding nucleo-mitochondrial interactions in mammalian cells is to identify the nuclear transcription factors that are common to the expression of these gene products. This has led to the purification and molecular cloning of nuclear respiratory factors, NRF-1 and NRF-2. The DNA binding and transcriptional specificities of these proteins have implicated them in the expression of many respiratory subunits along with key components of the mitochondrial transcription, replication, and heme biosynthetic machinery. In addition, tissue-specific transcription factors have been linked to the coordinate synthesis of contractile proteins and muscle-specific respiratory subunits whereas other more ubiquitous factors may have a dual function in nuclear and mitochondrial gene activation. These findings provide a framework for further investigations of the nuclear genetic mechanisms that integrate the expression of the respiratory apparatus with that of other cellular systems during growth and development.

KEY WORDS: ETS domain; gene expression; mammalian cells; mitochondria; nuclear respiratory factors; oxidative phosphorylation; regulation; respiratory chain; transcription.

INTRODUCTION

The essential role of the mitochondrion to cellular function has been highlighted in recent years by the discovery of genetic lesions that affect the respiratory apparatus and result in a variety of human diseases (Wallace, 1992; Larsson and Clayton, 1995). These generally manifest themselves as deficiencies in oxidative energy metabolism and most adversely affect those tissues that are most reliant on ATP production. The majority of these genetic defects have been ascribed to deletions, duplications, and point mutations in the mitochondrial genome. In mammals, the mtDNA genome is a small, covalently closed circular molecule with the capacity to encode 13 polypeptides. All of these polypeptides are subunits of the integral membrane complexes comprising the respiratory apparatus. The only other mitochondrial genes specify the 22 tRNAs and 2 rRNAs that are required for the translation of respiratory proteins within the mitochondrial matrix. Both RNA and protein-coding genes are the targets of mutations associated with human disease.

The limited coding capacity of the mitochondrial genome necessitates that nuclear genes contribute the majority of respiratory subunits and all of the proteins required for mitochondrial transcription, translation, and DNA replication. Despite the predominant role for nuclear gene products in the biogenesis and function of the mitochondrial respiratory chain, few nuclear genes have been implicated in mitochondrial genetic disorders. Genetic defects with a Mendelian inheritance pattern have been associated with syndromes involving the deletion of mtDNA (Zeviani *et al.*, 1989) and the reduction of mtDNA copy number (Moraes *et al.*, 1991). These observations suggest that affected individuals are defective in genes required for the replica-

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tion or maintenance of mtDNA. Although several components of the mitochondrial transcription and replication machinery have been isolated and cDNA clones obtained, their involvement in these disorders has not been established. Thus, little is known about the role of nuclear regulatory pathways in mitochondrial disease.

REGULATORY INTERACTIONS BETWEEN NUCLEAR AND MITOCHONDRIAL GENETIC SYSTEMS

Nucleus to Mitochondria

It has been well established that the regulated expression of nuclear genes in response to environmental signals is a key mechanism for mediating changes in respiratory chain function. In the yeast, *Saccharomyces cerevisiae*, the availability of oxygen and nonfermentable carbon sources regulates the expression of many nuclear genes encoding respiratory proteins (Zitomer and Lowry, 1992). This occurs via the activation or induction of specific transcription factors which are the targets of metabolic signaling pathways. Although the mechanisms are not well established in mammalian cells, numerous studies have described changes in the expression of nuclear respiratory genes in response to diverse signals. Such effectors of gene expression include hormones (Scarpulla *et al.*, 1986), cyclic nucleotides (Ku *et al.*, 1991), oncogenic transformation (Torroni *et al.*, 1990), and contractile activity (Williams *et al.*, 1987). Unidentified developmental signals also appear to induce the expression of tissue-specific isoform genes (Lomax and Grossman, 1989). In most of these cases, various respiratory chain subunits are induced at the mRNA or protein level but in most instances evidence for a specific transcriptional mechanism is lacking.

An exception is the induction of cytochrome *c* gene expression by cAMP. In this case a direct link has been established between the induction of cytochrome *c* mRNA by cAMP and the activation of gene transcription by the cAMP-dependent transcription factor, CREB (Evans and Scarpulla, 1989; Gopalakrishnan and Scarpulla, 1994). CREB binds to specific recognition sites in the cytochrome *c* promoter and activates transcription as a direct response to elevated cAMP levels. This up-regulation of cytochrome *c* may be a means by which certain hormones signal the availability of metabolic fuels to the respiratory apparatus. For example, glucagon and epinephrine stimulate adenylate cyclase activity through G-protein-linked receptors and thereby regulate the release of glucose from glycogen and the synthesis of glucose from amino acids (Linder and Gilman, 1992). Likewise, epinephrine directs the release of triacylglycerols, the storage form of fatty acids and a rich source of oxidative energy. Cytochrome *c* expression is also subject to transcriptional induction by thyroid hormone (Scarpulla *et al.*, 1986). However, this does not appear to involve direct activation of the promoter by thyroid hormone receptor, suggesting that other transcription factors serve as intermediaries in a thyroid-dependent pathway.

Mitochondria to Nucleus

A number of studies have investigated the nuclear response to mitochondrial impairment. This is best understood in yeast where the absence of mtDNA leads to the up-regulation of the nuclear gene encoding peroxisomal citrate synthase (Liao and Butow, 1993). This response, termed retrograde regulation, is mediated by transcription factors which recognize specific *cis*-acting promoter elements in nuclear target genes (Shyjan and Butow, 1993). In mammalian cells, perturbations in the expression of nuclear gene products have been linked to mtDNA mutations (Heddi *et al.*, 1993; Larsson *et al.*, 1994), the loss of mtDNA (Larsson *et al.*, 1994; Li *et al.*, 1995; Davis *et al.*, 1996), or with the inhibition of respiration or mitochondrial protein synthesis (Lunardi and Attardi, 1991; Chrzanowska-Lightowlers *et al.*, 1994). Certain ATP/ADP translocase isoform mRNAs (ANT2 and ANT3) are decreased when HL60 cells exit the cell cycle and undergo differentiation (Lunardi and Attardi, 1991). Similar reductions in the same transcripts occur in response to treatment of cells with a respiratory uncoupler or an inhibitor of mitochondrial protein synthesis. This contrasts with the increased expression of ANT1 and ATP synthase β-subunit mRNAs in the skeletal muscle of patients with respiratory defects resulting from mtDNA point mutations (Heddi *et al.*, 1993). Similar increases in nuclear mRNAs encoding cytochrome oxidase (COXIV and COXVIaL) and ATP/ADP translocase (ANT1 and ANT2) subunits were observed in ρ^0 cells completely lacking mtDNA (Li *et al.*, 1995). Both transcriptional (Lunardi and Attardi, 1991) and posttranscriptional (Chrzanowska-Lightowlers *et al.*,

1994) mechanisms have been advanced to explain the induction of nuclear mRNAs for respiratory proteins under conditions of impaired mitochondrial function.

Nuclear gene products required for mtDNA replication and transcription exhibit a differential response to the loss of mtDNA in ρ^0 cells. Mitochondrial DNA polymerase γ is expressed at similar levels in both ρ^0 and ρ^+ cells whereas mtTFA, a transcription factor that acts on mitochondrial promoters, is markedly reduced in ρ^0 cells (Larsson *et al.*, 1994; Davis *et al.*, 1996). Interestingly, mtTFA levels are reduced in the muscle fibers of patients with mtDNA depletion but increased in the fibers of patients with a mtDNA deletion that results in the abnormal proliferation of mtDNA (Larsson *et al.*, 1994). The reduction in mtTFA levels in ρ^0 cells is not accompanied by reduced mRNA levels. Thus, mtTFA is either regulated posttranscriptionally or the protein simply requires mtDNA for its stability. The initiation of mtDNA replication at the H-strand origin depends upon transcription from an mtTFA-dependent L-strand promoter. Therefore, a mechanism involving mtTFA in the regulation of mtDNA copy number is extremely attractive but thus far unproved.

NUCLEUS-ENCODED TRANSCRIPTION FACTORS ACTING ON NUCLEAR GENES

One approach to uncovering the nuclear regulatory mechanisms that control mitochondrial respiratory function is to identify the transcription factors that are common to the expression of nuclear respiratory genes. Transcription factors act as the targets of diverse signaling pathways and it is likely that they have an essential function in the pathways of nucleo-mitochondrial interaction in mammalian cells. With this rationale in mind, the rat cytochrome *c* gene was isolated and its promoter region characterized (Evans and Scarpulla, 1988, 1989). This gene has a complex promoter with multiple *cis*-acting elements dispersed over several hundred nucleotides. Among these are recognition sites for Sp1, CCAAT-box binding proteins, and, as discussed above, CREB.

In addition to these sites, a potent enhancer contains a specific binding site for a novel protein designated as nuclear respiratory factor 1 (NRF-1) (Evans and Scarpulla, 1989, 1990). As summarized in Table I, functional NRF-1 sites are found in many newly isolated genes encoding respiratory proteins, the rate-limiting enzyme in heme biosynthesis, and components of the mtDNA transcription and replication machinery (Chau *et al.*, 1992; Virbasius *et al.*, 1993a). A second transcription factor, designated as NRF-2, is required for maximal cytochrome *c* oxidase subunit IV (COXIV) and Vb (COXVb) promoter function (Virbasius and Scarpulla, 1991; Virbasius *et al.*, 1993b). As shown in Table I, these two proteins act on an overlapping subset of nuclear genes required for mitochondrial respiratory activity. Phylogenetic footprinting demonstrated that NRF-1 and NRF-2 binding sites in the COXVb promoter are conserved among rodent, human, and ten other primate genes (Bachman *et al.*, 1996). The prevalence of these factors in genes of related function led to the hypothesis that they contribute to the integrative expression of nuclear and mitochondrial genetic systems (Virbasius *et al.*, 1993a, b; Virbasius and Scarpulla, 1994; Scarpulla, 1996). In addition to NRF-1 and NRF-2, several other candidate factors for such a role have also been identified and will be discussed.

NRF-1

Functional NRF-1 sites have been identified in the majority of the characterized genes encoding subunits from cytochrome *c* reductase and oxidase complexes and the ATP synthase (Evans and Scarpulla, 1989, 1990; Chau *et al.*, 1992). In addition, the genes specifying the RNA subunit of the MRP endonuclease and mtTFA both depend upon functional NRF-1 sites for maximal promoter activity (Evans and Scarpulla, 1990; Virbasius and Scarpulla, 1994). As discussed below, mtTFA is a transcription factor that directs maximum expression from the divergent mitochondrial promoters within the mitochondrial D-loop regulatory region (Shadel and Clayton, 1993). The MRP endonuclease participates in the transition between transcription and replication by cleaving nascent light strand transcripts to generate primers for mitochondrial DNA replication (Clayton, 1992). As depicted in Fig. 1, these observations implicate NRF-1 as a potential link between the expression of respiratory subunits and the transcription and replication of mtDNA. NRF-1 has also been associated with the transcriptional expression of 5-aminolevulinate synthase (5-ALAS), the rate-limiting enzyme of heme biosynthesis within the mitochondrial matrix (Braidotti *et al.*, 1993). NRF-1 control over the synthesis of heme, an essential cofactor for the activity of respiratory cytochromes encoded by both genomes, is also indicative of a role for NRF-1 in intergenomic communication. Although NRF-1 activates transcrip-

Table I. Nucleus-Encoded Transcription Factors in Respiratory Chain Expression

Transcription factor and target genes	Reference
NRF-1	
rat somatic cytochrome *c*	(Evans and Scarpulla, 1989, 1990)
human somatic cytochrome *c*	(Evans and Scarpulla, 1990)
human ubiquinone binding protein	(Evans and Scarpulla, 1990)
human core protein 1	(U. Brandt, personal communication)
mouse cytochrome oxidase subunit Vb[a]	(Virbasius *et al.*, 1993a)
rat cytochrome oxidase subunit Vb[a]	(Bachman *et al.*, 1996)
human/primate cytochrome oxidase subunit Vb[a]	(Bachman *et al.*, 1996)
rat cytochrome oxidase subunit VIc	(Evans and Scarpulla, 1990)
bovine cytochrome oxidase subunit VIIaL[a]	(Seelan *et al.*, 1996)
bovine ATP synthase γ-subunit	(Chau *et al.*, 1992)
mouse MRP RNA	(Evans and Scarpulla, 1990)
human MRP RNA	(Evans and Scarpulla, 1990)
human mitochondrial transcription factor A[a]	(Virbasius and Scarpulla, 1994)
rat 5-aminolevulinate synthase	(Braidotti *et al.*, 1993)
NRF-2 (GABP)	
rat cytochrome oxidase subunit IV	(Virbasius and Scarpulla, 1991)
mouse cytochrome oxidase subunit IV	(Carter *et al.*, 1992)
mouse cytochrome oxidase subunit Vb[a]	(Virbasius *et al.*, 1993b)
rat cytochrome oxidase subunit Vb[a]	(Bachman *et al.*, 1996)
human/primate cytochrome oxidase subunit Vb[a]	(Bachman *et al.*, 1996)
bovine cytochrome oxidase subunit VIIaL[a]	(Seelan *et al.*, 1996)
human ATP synthase β-subunit	(Virbasius *et al.*, 1993b; Villena *et al.*, 1994)
human mitochondrial transcription factor A[a]	(Virbasius and Scarpulla, 1994)
mtTFA	
human/mouse mitochondrial heavy strand promoter	(Fisher *et al.*, 1987, 1989)
human/mouse mitochondrial light strand promoter	(Fisher *et al.*, 1987, 1989)
mTERF	
human mitochondrial transcription terminator	(Cooper *et al.*, 1993)
OXBOX/REBOX[b]	
human adenine nucleotide translocator muscle isoform	(Chung *et al.*, 1992; Haraguchi *et al.*, 1994)
human ATP synthase β-subunit	(Chung *et al.*, 1992; Haraguchi *et al.*, 1994)
Mt Element Binding Proteins[b]	
human ubiquinone binding protein	(Suzuki *et al.*, 1990)
human cytochrome c_1	(Suzuki *et al.*, 1990)
human ATP synthase β-subunit	(Suzuki *et al.*, 1990)
rat somatic cytochrome *c*	(Suzuki *et al.*, 1990)
human mitochondrial D-loop	(Suzuki *et al.*, 1995)
MEF-2/E-box	
mouse cytochrome oxidase subunit VIaH	(Wan and Moreadith, 1995)
rat cytochrome oxidase subunit VIII	(Lenka *et al.*, 1996)

[a] Genes contain functional recognition sites for both NRF-1 and NRF-2.
[b] Defined as DNA binding activities only.

tion through its specific binding sites in these genes, a regulatory function for the protein in cell signaling pathways has yet to be established.

NRF-1 has been purified to near homogeneity and a human cDNA clone isolated (Chau *et al.*, 1992; Virbasius *et al.*, 1993a). The recombinant protein displays the same binding and transcriptional specificities as the purified factor from HeLa cells (Virbasius *et al.*, 1993a). Antibodies directed against the recombinant protein recognize the specific NRF-1-DNA complexes formed with the purified HeLa protein or with crude nuclear extracts, further confirming the identity of the NRF-1 cDNA. Interestingly, NRF-1 is related through its DNA binding domain to developmental regulatory factors expressed in *Drosophila* and sea urchins. The sea urchin P3A2 factor functions in the site-specific transcriptional inhibition of a cytoskeletal actin gene whose expression is confined to the embryonic and larval ectoderm (Hoog *et al.*, 1991). The erect wing gene product (EWG) of *Drosophila* is expressed in the

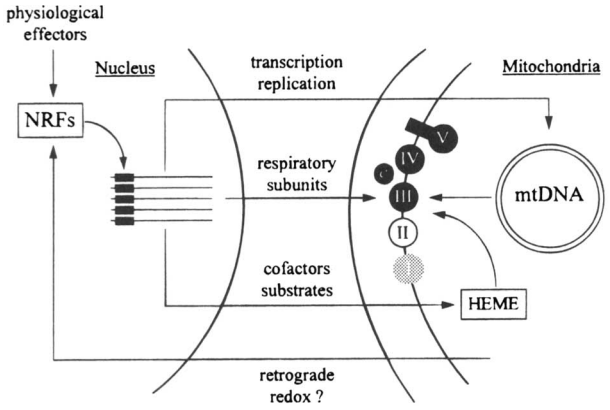

Fig. 1. Model for the nuclear control of the mitochondrial respiratory chain by nuclear transcription factors. Nuclear respiratory factors (NRFs: NRF-1 and/or NRF-2) contribute to respiratory chain expression and function by acting on nuclear genes encoding respiratory subunits, mitochondrial transcription and replication factors, and the rate-limiting heme biosynthetic enzyme. Complexes III, IV, and V and cytochrome *c* (solid spheres) have at least one subunit whose expression is under NRF control. It is unknown whether nuclear genes encoding complex I (shaded sphere) or II (open sphere) subunits have functional NRF sites in their promoters. NRFs may exert indirect effects on complexes I, III, IV, and V by acting on nuclear genes involved in the expression of mitochondrial DNA.

developing nervous system and is required for proper neuromuscular development (Desimone and White, 1993). Although localized to the nucleus, its transcriptional activity has not been demonstrated nor have target genes been identified. The high degree of sequence identity among the three proteins (NRF-1, P3A2, and EWG) is mainly confined to their novel DNA binding domains in keeping with the apparent differences in their biological functions (Virbasius *et al.*, 1993a). The human NRF-1 gene has been isolated and localized to q31 of chromosome 7 (Gopalakrishnan and Scarpulla, 1995).

Domain mapping experiments have revealed that NRF-1 binds DNA as a homodimer and has a modular structure that is characteristic of many transcription factors (Virbasius *et al.*, 1993a; Gugneja *et al.*, 1996). The transcription activation function resides in the carboxy-terminal end of the protein downstream from the DNA binding domain (Gugneja *et al.*, 1996). The activation domain is atypical in that it does not fall into the known classes defined by a preponderance of a particular amino residue. Instead, activation depends upon hydrophobic residues that are associated with specific hydrophobic clusters, each containing a single glutamine. Alanine substitution of the hydrophobic residues within key clusters inhibits the ability of NRF-1 to *trans*-activate a NRF-1-dependent promoter whereas alanine substitution for glutamine has no effect.

The amino-terminus upstream from the DNA binding domain has been resolved into two distinct subregions. The sequences proximal to the DNA binding domain comprise a complex nuclear localization signal that depends upon three clusters of basic residues for maximum function (Gugneja *et al.*, 1996). The second subregion in the amino terminus contains serine residues that are phosphorylated *in vivo* (S. Gugneja and R.C. Scarpulla, unpublished). The *in vivo* phosphorylation pattern can be replicated *in vitro* using casein kinase II, and the phosphorylated form of NRF-1 has enhanced DNA binding activity. This observation raises the possibility that NRF-1 binding is regulated by a phosphorylation-dependent pathway. Such a pathway may be used to modulate nuclear transcription in response to the availability of ATP, the major end-product of oxidative phosphorylation. Alternatively, the phosphorylated state of NRF-1 may be regulated by extracellular signals or in conjunction with the cell cycle. Experiments are under way to test these possibilities.

The discovery of functional NRF-1 sites in genes unrelated to the respiratory apparatus is suggestive of a broader integrative function (Chau *et al.*, 1992; Virbasius *et al.*, 1993a). A computer search has yielded a conservative estimate of about 50 target genes in addition to those shown in Table I. Several of these encode essential rate-limiting functions as well as proteins involved in cell growth, chromosome maintenance, and cell cycle regulation. Recently, the chicken homologue of NRF-1 has been associated with the expression of the histone *H5* gene during erythrocyte development (Gomez-Cuadrado *et al.*, 1995). A glycosylated version of the protein, termed chicken initiation binding repressor (cIBR), represses *H5* gene transcription *in vitro* whereas a nonglycosylated version, chicken initiation binding factor (cIBF), is present in transcriptionally active cells. cIBR is also phosphorylated but the specific functional consequences of either modification remains unknown. Thus, it is likely that NRF-1 and functionally related factors help integrate the expression of the energy-generating systems of mitochondria with a variety of other cellular activities.

NRF-2

NRF-2 was purified to near homogeneity based on its ability to activate COX gene expression through multiple GGAA sequence motifs (Virbasius *et al.*,

1993b). This motif is common to the recognition sites of the ETS-domain family of transcriptional activators which consists of lymphoid-specific and ubiquitously expressed members (Wasylyk *et al.*, 1993). The NRF-2 ETS sites are arranged tandemly in COXIV and COXVb promoters and behave synergistically in maximizing promoter activity (Virbasius and Scarpulla, 1991; Carter *et al.*, 1992; Virbasius *et al.*, 1993b; Carter and Avadhani, 1994). NRF-2 sites have also been observed in genes encoding COXVIIaL (Seelan *et al.*, 1996), the ATP synthase β-subunit (Virbasius *et al.*, 1993b; Villena *et al.*, 1994), and mtTFA (Virbasius and Scarpulla, 1994). In contrast to the homodimeric structure of NRF-1, NRF-2 is comprised of five subunits only one of which, the ETS-domain α-subunit, binds DNA by itself (Virbasius *et al.*, 1993b). The other four subunits, β_1, β_2, γ_1, and γ_2, associate with α in DNA–protein complexes and are most likely the alternative splice products of a single gene (Gugneja *et al.*, 1995).

Cloned cDNAs for all five NRF-2 subunits have been isolated and recombinant proteins expressed (Gugneja *et al.*, 1995). The results establish that NRF-2 is the human homologue of mouse GABP which was originally defined as a three-subunit activator of Herpes virus gene expression (LaMarco *et al.*, 1991; Thompson *et al.*, 1991). It is also identical to E4TF1, a multisubunit activator of adenovirus gene expression (Watanabe *et al.*, 1993). The two additional NRF-2 subunits (β_1 and γ_1) contain a small insertion of unknown function that is not present in the known mouse GABP homologues (Gugneja *et al.*, 1995). The NRF-2α subunit binds DNA and can heterodimerize with the β and γ subunits to form distinct DNA–protein complexes. The NRF-2β subunits have a carboxy-terminal homodimerization domain first observed in GABPβ_1 (Thompson *et al.*, 1991; Gugneja *et al.*, 1995). When complexed with α, these subunits confer high-affinity binding to tandemly arranged recognition sites, presumably through the formation of a tetramer. This can account for the high degree of transcriptional synergism observed among the NRF-2 sites present in COXIV and COXVb promoters (Virbasius and Scarpulla, 1991; Virbasius *et al.*, 1993b). The γ subunits, which differ from β only by the absence of the homodimerization domain, form lower-affinity complexes that have the same DNA binding specificity as the αβ complexes. Recent work has localized the NRF-2 transcriptional activation domain to a sequence near the carboxy-terminus that is common to the β and γ subunits. Thus, in addition to determining the DNA binding affinity on tandem sites, the non-DNA-binding subunits can deliver an activation domain to the DNA–protein complex. Interestingly, the NRF-2 activation domain is highly dependent on hydrophobic residues found in glutamine-containing hydrophobic clusters (Gugneja *et al.*, 1995, 1996). These motifs strongly resemble those required for transcriptional activation by NRF-1 (Gugneja *et al.*, 1996). This suggests that the factors contribute a hydrophobic activation surface in several genes (Table I, COXVb, COXVIIaL and mtTFA) where both NRF-1 and NRF-2 sites are in close proximity (Scarpulla, 1996).

Recently, NRF-2 (GABP) has been implicated in redox regulation. Treatment of cells with diethyl maleate, which depletes glutathione levels, decreases GABP binding activity without affecting the steady-state levels of the α subunit (Martin *et al.*, 1996). The activity of transfected promoters that depend upon GABP binding sites are also inhibited under these conditions. Binding activity is stimulated by physiological reducing agents *in vitro* and inhibited by oxidation. The observed redox effects are mediated by specific cysteine residues within the DNA binding and dimerization domains of the α subunit. In an independent study, a differential screening protocol identified COXIV among the mRNAs diminished upon diethyl maleate treatment (Ammendola *et al.*, 1995). These results suggest that NRF-2 (GABP) acts as a sensor to alter the expression of its target genes in response to the redox state of the cell. This is an attractive mechanism for regulating genes of the respiratory apparatus (Fig. 1). However, the conditions used in these studies are highly cytotoxic (Demonacos *et al.*, 1993), making it difficult to assess the regulatory significance of these findings.

It should be noted that NRF-2 (GABP) also acts on genes that are not related directly to respiratory metabolism. For example, the promoters of several ribosomal protein genes are dependent upon NRF-2 (GABP) sites for activity (Genuario *et al.*, 1993). Analysis of two of these genes has revealed both a site-specific activation and repression function for GABP that is promoter-dependent (Genuario and Perry, 1996). It is of interest in this context that the mRNA for ribosomal protein L4 is elevated in response to DEM treatment (Ammendola *et al.*, 1995). Although it is not known whether NRF-2 (GABP) acts on the *L4* gene promoter, the DEM effect may occur through alleviation of transcriptional repression.

Other Transcription Factors

A number of transcription factors have been implicated in the differential tissue-specific expression of nuclear respiratory genes. These have mainly been associated with the muscle-specific expression of tissue-specific isoform genes. The OXBOX/REBOX promoter element is present in the muscle-specific ADP/ATP translocator (ANT1) and in the ATP synthase β-subunit promoters (Li *et al.*, 1990; Chung *et al.*, 1992; Haraguchi *et al.*, 1994). The OXBOX element enhances transcription in muscle cells but not HeLa cells and binds proteins present only in myogenic cell lines. The overlapping REBOX element binds ubiquitous factors that are modulated in DNA binding *in vitro* by reducing agents and by thyroid hormone (Chung *et al.*, 1992). The *in vivo* significance of these observations has not yet been established. The specific identity of the OXBOX and REBOX binding proteins awaits their purification and molecular cloning.

Deletions within the muscle-specific COXVIa promoter were used to define a region that is lacking NRF-1, NRF-2, and OXBOX factor binding sites and is sufficient for myotube-specific expression (Wan and Moreadith, 1995). The tissue-specific expression directed by this region of the promoter depends upon MEF-2 and E-box consensus elements. The MEF-2 family of muscle-specific transcription factors is induced upon myogenic differentiation and has been implicated in the expression of contractile proteins (Olson, 1993). In a related study, the cardiac and skeletal muscle-specific expression of the COXVIII gene was attributed to two E-box elements in proximity to the transcription start site (Lenka *et al.*, 1996). As with COXVIaH, the COXVIII promoter is devoid of NRF-1, NRF-2, and OXBOX elements. Although the transcription factors responsible for tissue-specific expression have yet to be identified unambiguously, it is not surprising that they would be the same or similar to those governing the expression of other muscle-specific genes.

In contrast to COXVIaH and COXVIII, the promoter of the liver isoform COXVIIaL gene, which is expressed ubiquitously, is highly dependent on NRF-1, NRF-2, and Spl recognition sites (Seelan *et al.*, 1996). Purified recombinant NRF-1 and NRF-2 bind their cognate sites, and antibodies against the recombinant proteins recognize the DNA–protein complexes formed using nuclear extracts. These results are consistent with the observation that in gene pairs encoding ubiquitous and tissue-specific isoforms of a given protein, the NRF-1 site, when present, is associated with the ubiquitously expressed gene (Virbasius *et al.*, 1993a).

Finally, a series of conserved *cis*-acting promoter elements, termed Mt1, Mt3, and Mt4, is present in several genes including those for ubiquinone binding protein, cytochrome c_1, cytochrome *c*, and the ATP synthase β-subunit (Suzuki *et al.*, 1990). Four Mt element binding proteins have been detected by UV-induced DNA–protein crosslinking (Suzuki *et al.*, 1995), but the transcriptional activity and specificity of these proteins has not been established. An enhancer in the ATP synthase β-subunit gene that binds nuclear factors has also been described (Tomura *et al.*, 1990). These factors have not yet been characterized.

NUCLEUS-ENCODED TRANSCRIPTION FACTORS ACTING ON MITOCHONDRIAL GENES

mtTFA and mTERF

The transcription and replication of mtDNA in both simple and complex eukaryotes depends upon nucleus-encoded factors (Clayton, 1992; Jaehning, 1993; Shadel and Clayton, 1993). One well-characterized factor, mtTFA, was identified in mammalian cells and found to have functional homologues in yeast and amphibians (Jaehning, 1993; Shadel and Clayton, 1993; Antoshechkin and Bogenhagen, 1995). The protein stimulates transcription by mitochondrial RNA polymerase from divergent heavy (H) and light (L) strand promoters within the mitochondrial D-loop. The DNA binding and transcriptional activities of mtTFA are present in a single 25-kD protein which has an unusually high affinity for random DNA sequences (Fisher *et al.*, 1989). Sequencing and molecular cloning of the factor revealed an HMG box DNA binding domain in an otherwise unique sequence (Parisi and Clayton, 1991).

A similar HMG box protein, ABF2, is localized to yeast mitochondria and its absence results in the loss of respiratory activity and mtDNA in glucose-grown cells (Diffley and Stillman, 1991). Although ABF2 was unable to activate human L-strand transcription, both mtTFA and ABF2 can bend and unwind DNA and both bind yeast and human mtDNA promoter regions (Diffley and Stillman, 1992; Fisher *et al.*, 1992). A carboxy-terminal domain from human mtTFA can confer activation function on ABF2.

Moreover, expression of the human protein in yeast can restore respiratory competence and mtDNA levels to strains lacking ABF2 (Parisi *et al.*, 1993), demonstrating at least partial functional complementarity. The essential role of mtTFA in the transcription and maintenance of mtDNA makes it a prime candidate for a regulatory function in nucleo-mitochondrial interactions. However, there is currently no evidence that the factor is limiting for transcription *in vivo* or that nuclear control of mtDNA copy number is implemented by modulating the amount or activity of mtTFA.

As expected for a protein that functions in the mitochondria, the original human mtTFA cDNA encoded a mitochondrial targeting presequence that is not present in the mature protein (Parisi and Clayton, 1991). However, characterization of mouse mtTFA cDNA clones revealed the existence of multiple, alternatively-spliced transcripts (Larsson *et al.*, 1996). One of these encodes a testis-specific isoform that is lacking the mitochondrial targeting sequence. This transcript gives rise to a slightly larger variant of mtTFA that is localized to the nuclei of spermatocytes and elongating spermatids. Although no target genes have been identified, this finding raises the interesting possibility that mtTFA may function as a nuclear transcription factor in certain cell types. However, its relatively high abundance and localization to elongating spermatids, where no new transcription occurs, is also consistent with a structural role.

Another nucleus-encoded factor functions in the termination of mitochondrial transcription. Many of the heavy strand transcripts terminate at the junction between 16S rRNA and leucyl-tRNA genes at a bidirectional termination site. The mitochondrial termination factor mTERF was initially identified as an activity that binds this site and promotes termination *in vitro* (Kruse *et al.*, 1989). The binding activity was subsequently associated with several related polypeptides of 31 or 34-kD that copurify on DNA affinity chromatography (Daga *et al.*, 1993). The termination activity, however, resides only in the 34-kD species. Interestingly, a point mutation linked to MELAS (mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes) diminishes the termination of 16S rRNA transcription *in vitro* and reduces the binding affinity of a partially purified 34-kD protein (Hess *et al.*, 1991). These findings are suggestive of a potential link between human disease and the activity of a nucleus-encoded regulatory factor. The precise structural relationships among these proteins and their *in vivo* functions await more detailed molecular and genetic analysis.

Factors Acting on Both Nuclear and Mitochondrial Genes

In several cases it has been proposed that nuclear and mitochondrial genes are controlled coordinately by common *cis*-acting elements that are the targets of the same or similar transcription factors. Sequence similarities to the OXBOX/REBOX (Haraguchi *et al.*, 1994) and Mt (Suzuki *et al.*, 1991, 1995) elements have been localized to the mitochondrial D-loop. The ability of these elements and their nuclear gene counterparts to bind proteins from crude extracts with the same specificity has been taken as evidence for shared regulatory factors between the two genetic systems (Suzuki *et al.*, 1991; Haraguchi *et al.*, 1994). It is of interest in this context that two of the five regions of DNA–protein interaction detected in mtDNA *in vivo* have sequence similarities to the Mt4 element (Ghivizzani *et al.*, 1993; Suzuki *et al.*, 1995). One of these coincides with an mtTFA binding site and the other with a conserved sequence block near the region of transition between transcription and replication. However, aside from their DNA binding activities, there is no evidence to support a functional role for the Mt element binding proteins in the expression of either nuclear or mitochondrial genes.

Binding sites for ligand-dependent *trans*-activators have also been observed in mtDNA (Demonacos *et al.*, 1995; Wrutniak *et al.*, 1995). A 43-kD protein, closely related to the c-Erb A α1 thyroid hormone receptor, has been localized to the mitochondrial matrix and observed to bind the mitochondrial D-loop *in vitro* (Wrutniak *et al.*, 1995). This protein was proposed to be an amino-terminally truncated variant of the nuclear α1 thyroid hormone receptor, but this was not confirmed by structural analysis of the purified mitochondrial receptor. Nevertheless, the overexpressed variant gave a particulate cytosolic immunofluorescence and stimulated mitochondrial respiration as measured by rhodamine staining and cytochrome oxidase activity. Similarly, the rat liver glucocorticoid receptor (GR) was found to translocate to the mitochondria upon treatment of animals with dexamethasone (Demonacos *et al.*, 1993). Six glucocorticoid response elements (GREs) were found in mtDNA and were shown to bind GR *in vitro* (Demonacos *et al.*, 1995). Two of the six elements

are present in the D-loop but the other four are within the COXI and COXIII genes, far removed from the *in vivo* sites of mtDNA transcription initiation. These cases of regulatory factors shared between nucleus and mitochondria rest primarily upon DNA binding as the only functional assay. It is important to note that the transcriptional activity and specificity of the putative *cis*-elements and *trans*-acting factors using mtRNA polymerase and defined mtDNA templates has not been demonstrated.

PERSPECTIVE

Much progress has been made in recent years on the nuclear control of respiratory chain expression in mammalian cells. It is evident that the nuclear genes involved are controlled by a diverse collection of regulatory proteins that are not unique to this function. It is likely that the energy-generating systems of mitochondria are integrated at the transcriptional level with many other cellular systems. This is evident in the utilization of CREB for the cAMP control of cytochrome *c* expression and of MEF-2 and E-box factors for the muscle-specific expression of COX subunits. The combinatorial action of transcription factors within a given promoter context may also serve as a major determinant of physiological function.

As summarized in Table I, many of the known nuclear genes whose products contribute to the respiratory apparatus are the targets of NRF-1 and NRF-2, and a subset of these genes is dependent on both factors. These findings have led to the model depicted in Fig. 1. A key feature of this model is that NRFs, and related factors, act on nuclear genes to bring about the synthesis of respiratory subunits from both genetic systems. This would occur via the coordinate transcriptional expression of respiratory chain subunits along with key transcription and replication factors (mtTFA and MRP endonuclease) and the rate-limiting heme biosynthetic enzyme (5-aminolevulinate synthase). This model is consistent with the DNA binding and transcriptional activities of the purified proteins on well-characterized target genes but does not account for the subset of genes whose promoters lack recognition sites for NRF-1 or NRF-2. Perhaps only those components that are rate-limiting for the assembly of respiratory complexes, for electron transfer, or for certain catalytic functions are subject to coordinate transcriptional control. It is also possible that these NRF-dependent pathways operate in conjunction with transcription factors that are shared between the two genetic systems. However, the case for shared factors remains highly speculative. An essential proof of the validity of this model requires the genetic manipulation of the activity or amounts of NRFs *in vivo*.

A key issue is whether NRFs and related factors can respond to extra- and intracellular signals to bring about regulated expression of the respiratory apparatus. Several possibilities have been highlighted by recent findings. The fact that *trans*-activation and DNA binding functions are on different NRF-2 subunits (Gugneja *et al.*, 1996) raises the prospect that regulation occurs through subunit associations. Such a mechanism has been observed for carbon source regulation of respiratory genes through the yeast activator HAP2/3/4/5 (McNabb *et al.*, 1995). In this case a heterotrimeric DNA binding complex (HAP2/3/5) associates with an inducible subunit (HAP4) that contains the transcriptional activation domain (Forsburg and Guarente, 1989). As discussed above, NRF-2 may be subject to a retrograde pathway involving redox regulation of DNA binding (Martin *et al.*, 1996). Post-transcriptional mechanisms may also come into play. The phosphorylation of NRF-1 affects its DNA binding activity *in vitro* and may be utilized for extra- or intracellular signaling *in vivo*. Finally, preliminary experiments suggest that NRF-1 mRNA is highly induced in electrically stimulated cardiac myocytes where mitochondrial proliferation is evident (J. McMillin, personal communication). Further investigations of these mechanisms should yield new insights into the pathways of nucleo-mitochondrial communication in mammalian systems and their potential role in human disease.

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