Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: A potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis

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Communicated by E. Margoliash, October 18, 1993

ABSTRACT Mitochondrial transcription factor A (mtTFA), the product of a nuclear gene, stimulates transcription from the two divergent mitochondrial promoters and is likely the principal activator of mitochondrial gene expression in vertebrates. Here we establish that the proximal promoter of the human mtTFA gene is highly dependent upon recognition sites for the nuclear respiratory factors, NRF-1 and NRF-2, for activity. These factors have been previously implicated in the activation of numerous nuclear genes that contribute to mitochondrial respiratory function. The affinity-purified factors from HeLa cells specifically bind to the mtTFA NRF-1 and NRF-2 sites through guanine nucleotide contacts that are characteristic for each site. Mutations in these contacts eliminate NRF-1 and NRF-2 binding and also dramatically reduce promoter activity in transfected cells. Although both factors contribute, NRF-1 binding appears to be the major determinant of promoter function. This dependence on NRF-1 activation is confirmed by in vitro transcription using highly purified recombinant proteins that display the same binding specificities as the HeLa cell factors. The activation of the mtTFA promoter by both NRF-1 and NRF-2 therefore provides a link between the expression of nuclear and mitochondrial genes and suggests a mechanism for their coordinate regulation during organelle biogenesis.

Mitochondrial biogenesis in eukaryotic cells has a requirement for gene products from two physically separated genomes: one contained within the organelle and the other within the nucleus. Recent developments in understanding the mechanisms of mitochondrial RNA replication and transcription have led to the identification of essential nuclear-encoded components (for review, see ref. 1). Among these is mitochondrial transcription factor A (mtTFA; previously known as mtTF1) (2, 3). This protein binds to sequence elements in the divergent heavy- and light-strand promoters within the mitochondrial D-loop region and stimulates transcription from mitochondrial DNA templates in vitro (2, 3). In support of its in vivo function, expression of human mtTFA in yeast restores both mitochondrial DNA and respiratory competence to strains lacking the yeast homolog (4, 5). Since transcripts initiating at the light-strand promoter provide primers for heavy-strand replication (the essential first step of mitochondrial DNA replication), mtTFA may provide an important control point for both mitochondrial DNA copy number and transcriptional activity (1). By extension, the nuclear origin of mtTFA makes it an attractive candidate for a link between nuclear and organelar gene expression. The recent report of the nucleotide sequence of the mtTFA gene (6) has provided an opportunity to examine the mechanisms governing its expression. We report here that the proximal promoter of the mtTFA gene is highly dependent on nuclear respiratory factors (NRF) 1 (7-9) and 2 (10, 11).

Functional recognition sites for these nuclear transcription factors have been found in a number of nuclear genes whose products contribute to mitochondrial respiratory function (7-11). These include genes encoding cytochrome c, subunits for three of the five respiratory complexes, and the mitochondrial RNA processing RNA (7, 8). The latter is the nucleic acid moiety of an enzyme that can cleave light-strand transcripts to generate RNA primers for mitochondrial heavy-strand DNA replication (12-14). Recently, NRF-1 has also been implicated in the expression of 5-aminolevulinate synthase, the rate-limiting enzyme in the synthesis of heme for respiratory cytochromes (15). NRF-2 was identified as an activator of cytochrome oxidase gene expression and has been purified to near homogeneity from HeLa cells (11). This multisubunit activator shares several homologous subunits with the mouse GA-binding protein, an Ets-domain transcription factor involved in the expression of Herpes simplex virus immediate early genes (16, 17). We propose that via their activation of the mtTFA gene, NRF-1 and NRF-2 have the potential to communicate nuclear regulatory events to the mitochondrial transcription and replication machinery.

MATERIALS AND METHODS

Synthesis of the mtTFA Promoter. The mtTFA sequence from −86 to +20 was synthesized as a series of overlapping double-strand oligonucleotides (the NRF-1 and NRF-2 target sequences are in boldface type):

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>GATCCCGGGGTATA GGGCCCATGCGAGA</td>
</tr>
<tr>
<td>2</td>
<td>CCCTCTCCCGCCGTTCGGCGCAATT GGGCGCCGAGCGGTTAGGCGGG</td>
</tr>
<tr>
<td>3</td>
<td>CCGGGGCGCCGCCCCCAGGAGCGATCACC</td>
</tr>
<tr>
<td>4</td>
<td>TCTACCGAAGCCTGTTAGTGGC</td>
</tr>
<tr>
<td>5</td>
<td>AGATTTCCATAGTGGCTCCTAGTGGCGGGCATG GGGATACGGAGCGGATCACC</td>
</tr>
<tr>
<td>6</td>
<td>GCGCGTACCAGT</td>
</tr>
</tbody>
</table>

These were phosphorylated, annealed, and ligated, and the synthetic fragment was purified and ligated in turn to a promoterless chloramphenicol acetyltransferase (CAT) expression cassette as described (11). Mutations in factor binding sites (lowercase letters) were incorporated by substituting the following oligonucleotides for the above:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M</td>
<td>CGCTCTCCCGCtaCTatGCCAATT GGGCGatGtaCGGTTAAGCGGG</td>
</tr>
</tbody>
</table>

Abbreviations: NRF, nuclear respiratory factor; mtTFA, mitochondrial transcription factor A; RC4, rat cytochrome c; CAT, chloramphenicol acetyltransferase.

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Versions of oligonucleotides 2 and 4 above were prepared with BamHI- and HindIII-compatible overhangs for cloning into the truncated rat cytochrome c (RC4) promoter vector pRC4CATBΔ/-66BΔ as described for analysis of the NRF-1 and NRF-2 binding sites in transcription reactions (8, 11).

**Proteins and DNA-Binding Assays.** The purification of HeLa NRF-1 (9) and NRF-2 (11) and the cloning and expression of recombiant NRF-1 (18) have been described. The cloning and expression of the α and β2 subunits of human NRF-2 (11), the human homologues of rodent GA-binding protein α and β1 (17), will be described elsewhere (S. Gugneja, J.V.V., and R.C.S.). Recombinant NRF-2 contains equimolar amounts of bacterially expressed human α and β2 subunits. Recombinant Sp1 was from Promega. Electrophoretic mobility shift, methylation interference, and DNase I footprinting assays were performed as described (7, 8).

**Transfections.** Growth of COS-1 cells and transfection by the calcium phosphate method were as described (19). Two days after transfection, cells were harvested, protein extracts were assayed for CAT activity, and HiT supernatant DNA was assayed for CAT coding sequence. CAT activities are normalized to the CAT DNA content to correct for differences in transfection efficiency, and results represent the average of four separate determinations.

**In Vitro Transcription.** Preparation of HeLa nuclear extracts, transcription reactions, and assay of products by primer extension have been described (11). The radiolabeled primer is complementary to the first exon of the RC4 gene and results in a 61-nt CDNA product from RC4 promoter constructions and a 67-nt product from RNAs initiated at the major mtTFA start site (6).

**RESULTS**

Inspection of a recently published genomic sequence of the 5′-flanking region of the mtTFA gene (6) revealed potential binding sites for NRF-1 and NRF-2. These flanked several overlapping Sp1 consensus binding sequences (20) in a region of about 60 bp (Fig. 1). To assess the function of these sites, the putative mtTFA promoter region from −86 to +20 was synthesized from a series of overlapping oligonucleotide modules and fused to the first exon of a cytochrome c–CAT fusion gene. In this construct the cytochrome c promoter and transcription start sites were replaced with those resident in the mtTFA sequence. Versions containing mutations in each of the potential factor binding sites were also prepared.


**Fig. 1.** Summary of NRF-1 Sp1, and NRF-2 recognition sites and their effects on mtTFA promoter function. The sequence of the mtTFA gene from −80 to +11 is shown at top. Brackets indicate regions protected from DNase I digestion by binding of the indicated proteins, whereas solid triangles indicate enhanced DNase I cleavages. Solid circles denote strong guanine nucleotide contacts, and open circles denote residues where partial interference was detected by methylation interference footprinting shown in Fig. 3. The mtTFA–CAT reporter construction is shown schematically, and normalized CAT activities of the various mutant constructions are given at right (expressed as average ± SD of four determinations, with the activity of the wild-type construct defined as 1.0).
that DNA sequences with the GGAT in NRF-2 of this technique, well targets recognition guanine several promoters mtTFA GCGC motifs interfered binding excess at native gels. Where indicated (+) reactions also included a 200-fold excess of unlabeled NRF-1 (RC4 – 172/–147) or NRF-2 (RC04 +13/+36) binding site oligonucleotides. Products were separated on native acrylamide gels and detected by autoradiography.

Fig. 2. Specific binding of affinity-purified HeLa NRF-1 and NRF-2 to the mtTFA promoter. A labeled DNA fragment including the entire synthesized mtTFA promoter sequence (lanes 1–6) or mutant versions with disrupted NRF-1 (mut 1; lanes 7–12), Spl (mut 2; lanes 13–18), or NRF-2 (mut 3; lanes 19–24) binding sites were incubated with ~2 ng of purified HeLa NRF-1 or 5 ng of NRF-2 as indicated. Where indicated (+) reactions also included a 200-fold excess of unlabeled NRF-1 (RC4 – 172/–147) or NRF-2 (RC04 +13/+36) binding site oligonucleotides. Products were separated on native acrylamide gels and detected by autoradiography.

guanine nucleotide contacts made by NRF-1 and NRF-2 upon binding the mtTFA promoter (Fig. 3). As previously observed for more than 10 different NRF-1 binding sites (8, 9), methylation at each of the guanine nucleotides in the tandem GCGC motifs interfered with NRF-1 binding to the putative mtTFA promoter site (summarized in Fig. 1). Extended contacts toward the 3' end of the lower strand are unusual but are similar to the contacts made by NRF-1 upon binding the cytochrome c promoter (8). Several binding sites for NRF-2 as well as other Ets-domain proteins have been analyzed by this technique, and all share contacts to the guanine nucleotides of the central GGAA/T motif of their recognition sites (10, 11, 21). Similarly, methylation of the guanine nucleotides in the GGAT sequence in the mtTFA promoter eliminates that DNA from the fraction bound by NRF-2. In agreement with previously analyzed sites, no guanine contacts on the lower strand were discovered. These results demonstrate that the sequences resembling the consensus for NRF-1 and NRF-2 in the mtTFA promoter are in fact functional as recognition targets for those proteins in vitro.

Mutations in NRF-1 and NRF-2 sites in the context of several promoters have been shown to affect the activity of these promoters in cultured cells (7, 8, 10, 11, 15). To investigate the contribution of the sites to mtTFA promoter function, the CAT activities from expression vectors with nucleotide substitutions in the NRF-1, NRF-2, and Spl sites, either singly or in various combinations, were compared to the wild-type mtTFA promoter (Fig. 1). A mutation that eliminates NRF-1 binding to the mtTFA promoter fragment (mut 1) results in a drastic decrease in activity, indicating that NRF-1 binding plays a major role in the activation of this minimal promoter. Mutations in either the Spl or NRF-2 sites (mut 2 and mut 3, respectively) result in more modest but significant decreases in activity, indicating that these sites are also functional within the mtTFA promoter context. Combined mutations in the Spl and NRF-2 binding sites (mut 2,3) resulted in the expected activity reduction predicted from the combined effect of the two substitutions. However, when the NRF-1 site was already mutated, mutation of the Spl or NRF-2 sites did not significantly reduce activity compared to the NRF-1 mutation alone (compare mut 1, 2 or mut 1, 3 with mut 1). This result suggests that activation by Spl and NRF-2 requires the presence of a functional NRF-1 binding site and points to NRF-1 as a key activator of the mtTFA promoter. Combined mutation of all three sites eliminated >95% of the promoter activity (compare mut 1,2,3 with wt), and thus these sites are essential to the proximal mtTFA promoter in transfected cells.

Multiple molecular species could be responsible for transcriptional activation through a given site in transfected cells. We have recently isolated recombinant clones encoding proteins with the DNA binding and transcriptional specificities of NRF-1 (18) and NRF-2 (S. Gugnjea, J.V.V., and R.C.S., unpublished results). The recombinant proteins were thus used to establish a direct connection between the site-specific binding and transcriptional activation of the mtTFA promoter. Recombinant NRF-1 and NRF-2 were over produced in Escherichia coli and purified to near homogeneity, whereas recombinant Spl was obtained from a commercial source. These proteins were tested for specific binding to the mtTFA promoter by DNase I footprinting assays (Fig. 4).

Binding of recombinant NRF-1 to an mtTFA promoter fragment protects a region corresponding to the area of guanine nucleotide contacts of the purified NRF-1 preparation (lanes 3, 13, and 18). Additionally, NRF-1 binding results
in a strong enhancement of cleavages at the 5' end of its binding site as previously observed for other NRF-1 sites (7, 8). No protection or enhancement by NRF-1 is observed on the mut 1 fragment, demonstrating the specificity of binding as well as the effectiveness of the mutation. Nearly abutting the NRF-1 footprint is a region extending over two of the putative Sp1 sites that is protected from cleavage by Sp1 binding (lanes 4, 9, and 19). This protection is also eliminated by the mut 2 mutation (lane 14), arguing that the transcriptional effects of this mutation are due, at least in part, to disruption of Sp1 binding. Finally, extending from the Sp1 footprint to a location = 12 bp from the major transcription start site is a region protected by NRF-2 binding (lanes 5, 10, and 15). As predicted, the mut 3 mutation eliminates the NRF-2 footprint obtained with the recombinant protein (lane 20). Together, these proteins alter the DNase I cleavages in a nearly uninterrupted region from about -80 to -10 (summarized in Fig. 1).

We also tested binding of mixtures of the recombinant proteins (data not shown) but observed no cooperativity at the level of DNA binding. We presume that the dependency on NRF-1 binding for activation by Sp1 and NRF-2 operates through a mechanism other than direct interaction among these proteins, perhaps requiring formation of complexes with an additional factor. However, since recombinant NRF-1 and NRF-2 were purified from bacteria, we cannot rule out a protein modification by mammalian cells that would potentiate direct interaction and synergism in binding.

The recombinant NRF-1 and NRF-2 proteins have been shown to activate transcription in vitro from known target templates. To establish a direct connection between recognition of the mtTFA promoter sites by these proteins and transcriptional activation, in vitro transcription reactions were carried out using mtTFA promoter templates (Fig. 5). In agreement with the relatively low activity of this promoter in transfected cells, the wild-type template in the absence of added NRF-1 gave a low but detectable level of transcription, resulting in a primer extension product of exactly the size expected for fusion transcripts initiating at the native mtTFA transcription start site (Fig. 5A, lane 2). Addition of only 100 ng of purified recombinant NRF-1 protein dramatically enhanced the mtTFA transcript (lane 3). No further activation was observed with increased addition of NRF-1 (lanes 4 and 5). A transcript of the expected size was not detected from the mut 1 template even when recombinant NRF-1 was included in reaction mixtures (lanes 6–9). This is perhaps not surprising given the 10-fold lower activity of this template in transfected cells, and in a 60-min in vitro transcription reaction accumulation of transcripts apparently does not reach a detectable threshold. This result does, however, verify the importance of the NRF-1 site in the function of this promoter.

The absence of a detectable transcript renders the mut 1 template unsatisfactory as a negative control for stimulation by NRF-1. The experiment was therefore repeated using the RC4 promoter with a linker insertion known to disrupt the NRF-1 binding site (7) as an internal control template (Fig. 5B). As above, addition of recombinant NRF-1 stimulates transcription from the wild-type mtTFA template (lanes 1–4),
achieving a plateau of activity with the addition of 100 ng of NRF-1, whereas no transcript from the mutant 1 template was detected in either the presence or absence of added NRF-1 (lanes 5–8). The reference RC4 transcript in either case is unaffected by the addition of NRF-1, confirming the specificity of the stimulation for a template containing a functional target site. Therefore, the requirement for NRF-1 and its recognition site to obtain detectable levels of mtTFA transcripts in vitro accurately reflects the requirement for a NRF-1 site for mtTFA promoter activity in transfected cells.

In similar experiments, the mtTFA template was not stimulated by NRF-2 to a level reproducibly detectable in this assay. This may in part be a technical problem of measuring weak promoter activity in vitro. However, we cannot exclude the possibility that the nuclear extracts are limiting in a component required for maximal activation by NRF-2 within the mtTFA promoter context. To verify the function of the mtTFA NRF-1 and NRF-2 sites in an identical context, they were cloned in the same position upstream of a truncated RC4 promoter and tested for their ability to direct activated transcription in vitro (Fig. 5 C and D). The truncated promoter (RC4CAT–/–66) has neither NRF-1 nor NRF-2 binding sites and as expected was not affected by the addition of either recombinant NRF-1 (Fig. 5C, lanes 1–3) or NRF-2 (Fig. 5D, lanes 1–3). However, when the mtTFA NRF-1 site oligonucleotide (mtTFA–76–/–58) was cloned upstream, transcription was substantially enhanced (Fig. 5C, lanes 4–6), confirming the ability of NRF-1 to stimulate through this sequence. Similarly, the NRF-2 recognition sequence from the mtTFA promoter (mtTFA–34–/–13) confers responsiveness to the addition of recombinant NRF-2 to this construct (Fig. 5D, lanes 4–6). Taken together, these results demonstrate that both NRF-1 and NRF-2 participate in the activation of the mtTFA promoter by direct stimulation through their respective binding sites.

**DISCUSSION**

The initial finding of NRF-1 and NRF-2 recognition sites in nuclear genes that specify mitochondrial respiratory proteins suggested that they participate in the coordinate expression of these genes (7–11). While control of such nuclear genes through common regulatory factors may be useful, such a mechanism does not account for their concerted expression with mitochondrial genes encoding respiratory subunits. NRF-1 and NRF-2 would play a more integrative role in nuclear–mitochondrial interactions if they also governed the transcription of nuclear genes required for either respiratory organelle function or the expression of the mitochondrial subunits. Those genes that specify components of the mitochondrial transcription and replication machinery would be attractive candidates for regulation. In fact the discovery of functional NRF-1 sites in both mouse and human genes encoding the mitochondrial RNA-processing RNA led to the prediction that other genes of this type would be targets for NRF-1 activation (8).

Moreover, NRF-1 sites have been recently found to dramatically influence the expression of the gene encoding 5-aminolevulinate synthase, the rate-limiting enzyme in the synthesis of heme for respiratory cytochromes (15). Thus, by acting on this gene, NRF-1 may indirectly coordinate the activities of respiratory subunits encoded by both genomes. Here we provide evidence that NRF-1 and NRF-2 are major determinants of mtTFA gene transcription. How might this activation provide a link to regulatory events directly affecting the organelle? First, mtTFA binds to elements in both heavy- and light-strand promoters and appears to be the principal activator of transcription by the mitochondrial RNA polymerase (1). NRF-dependent modulation of the amount of mtTFA present in the mitochondria may directly affect the rate of transcription and therefore the abundance of the respiratory subunits, rRNAs, and tRNAs encoded in the mitochondrial genome. Second, since transcripts from the light-strand promoter provide primers for heavy-strand DNA replication, the rate of transcription, as determined by mtTFA, may affect copy number of the mitochondrial DNA (1).

We should note that as yet there is no direct proof of a relationship between mtTFA expression and the rate of mitochondrial transcription or replication. Recently, it has been reported that levels of mtTFA mRNA are not changed in cultured cell lines lacking mitochondrial DNA or with a defective mitochondrial genome (22). This result appears inconsistent with the regulation of mtTFA expression in response to oxidative phosphorylation-dependent signals emanating from the mitochondria. However, such cells are provided with sufficient nutritional sources to generate abundant glycolytic energy for continued growth in the absence of a functional respiratory chain. Under these conditions, a signaling pathway that would normally respond to a deficiency in respiratory energy production may not be activated.

Regardless of the role of mtTFA in the regulated expression of respiratory subunits, mechanisms must exist for the maintenance of mitochondrial DNA. It is of interest in this context that disruption of the gene that encodes the homolog of mtTFA in yeast leads to a loss of both mitochondrial DNA and respiratory function (23). NRF-1 and NRF-2 may help maintain mitochondrial DNA copy number by transcribing cell cycle regulatory signals acting on the nucleus to the mitochondrial transcription and replication machinery through their effects on nuclear gene expression. In addition, effectors such as thyroid hormones, which regulate mitochondrial biogenesis and function (for references, see ref. 24), may do so by controlling NRF gene expression or biological activity. Similarly, NRFs may respond to cytoplasmic signals to communicate the energetic state of the cytoplasm back to the nuclear transcriptional apparatus. The molecular cloning of these factors will open the way to addressing these possibilities.

This work was supported by U.S. Public Health Service Grant GM 32525-10 from the National Institutes of Health. R.C.S. is the recipient of Faculty Research Award FRA-361 from the American Cancer Society.