Nuclear Respiratory Factor 1 Activation Sites in Genes Encoding the 
γ-Subunit of ATP Synthase, Eukaryotic Initiation Factor 2α, and 
Tyrosine Aminotransferase

SPECIFIC INTERACTION OF PURIFIED NRF-1 WITH MULTIPLE TARGET GENES*

(Received for publication, June 17, 1991)

Ching-man A. Chau, Mark J. Evans+, and Richard C. Scarpull‡

From the Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School, Chicago, Illinois 60611

Transcription factor nuclear respiratory factor 1 (NRF-1) was originally identified as an activator of the cytochrome c gene and subsequently found to stimulate transcription through specific sites in other nuclear genes whose products function in the mitochondria. These include subunits of the cytochrome oxidase and reductase complexes and a component of the mitochondrial DNA replication machinery. Here we establish that a functional recognition site for NRF-1 is present in the ATP synthase γ-subunit gene extending the proposed respiratory role of NRF-1 to complex V. In addition, biologically active NRF-1 sites are found in genes encoding the eukaryotic translation initiation factor 2 α-subunit and tyrosine aminotransferase, both of which participate in the rate-limiting step of their respective pathways of protein biosynthesis and tyrosine catabolism. The recognition sites from each of these genes form identical complexes with NRF-1 as established by competition binding assays, methylation interference footprinting, and UV-induced DNA crosslinking. Cloned oligomers of each NRF-1 binding site also stimulate the activity of a truncated cytochrome c promoter in transfected cells. The NRF-1 binding activities for the various target sites copurified approximately 33,000-fold and resided in a single protein of 68 kDa. These observations further support a role for NRF-1 in the expression of nuclear respiratory genes and suggest it may help coordinate respiratory metabolism with other biosynthetic and degradative pathways.

Much of what is known about the control of gene expression in vertebrate organisms comes from studying highly inducible genes, most of which encode the specialized products of terminally differentiated cells. By contrast, little is understood of the genetic regulation of metabolic systems required by all animal cells during growth and development. The control of respiratory chain biosynthesis is of particular interest because it requires the concerted modulation of a large number of genes encoded by both nuclear and mitochondrial genetic compartments (1, 2). In mammals, mitochondrial genes contribute 13 respiratory subunits along with the ribosomal and transfer RNAs required for mitochondrial translation (1). Nuclear genes encode the majority of the respiratory protein subunits and all of the enzymes and structural proteins required for mitochondrial transcription, translation, and replication. Since the products of both genomes are essential to respiratory function, mechanisms must exist for their coordination in response to cellular energy demands.

We have isolated and characterized the rat (3, 4) and human (5) cytochrome c genes with the expectation of uncovering control elements common to other respiratory chain genes. These studies have led to the identification of multiple cis-acting elements of potential regulatory significance (6, 7). The best characterized of these is the recognition site for NRF-1, an activator protein which stimulates transcription through specific sites found in several recently isolated nuclear genes encoding mitochondrial functions (7, 8). Two of these genes specify subunits of the cytochrome c reductase (Ref. 9, ubiquinone-binding protein) and oxidase (Ref. 10, subunit Vlc) complexes. A third gene encodes the RNA subunit of MRP endonuclease, a ribonucleoprotein enzyme which generates primers for mitochondrial DNA replication (11–13). The presence of functional NRF-1 sites in these genes is suggestive of a role for NRF-1 in modulating respiratory chain function (14).

Here we describe the purification of NRF-1 to near homogeneity and identify three additional target genes for NRF-1 activation by establishing that a functional recognition site for the factor resides in each. The presence of a NRF-1 site in the gene for the ATP synthase γ-subunit (γ-ATP synthase) of complex V (15) lends further credence to a role for NRF-1 in respiratory chain expression. Moreover, NRF-1 sites are also found in genes encoding the eukaryotic initiation factor 2 α-subunit (eIF-2α) (16) and tyrosine aminotransferase (17). Both of these proteins are engaged in the rate-limiting step of their respective pathways of protein synthesis (18) and tyrosine catabolism (19). These observations are indicative of respiratory chain biosynthesis. The abbreviations used are: NRF-1, nuclear respiratory factor 1; γ-ATP synthase, ATP synthase γ-subunit; eIF-2α, eukaryotic initiation factor 2 α-subunit; RC4, rat somatic cytochrome c gene; ATF, activating transcription factor; COXVI-c, cytochrome oxidase subunit Vlc gene; mMRP, mouse mitochondrial RNA processing; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
a more global function for NRF-1 in integrating respiratory activity with other pathways which either consume respiratory energy or generate the required substrates.

**EXPERIMENTAL PROCEDURES**

**Synthetic Oligonucleotides**—The following synthetic DNA oligomers were utilized for DNA binding and transfection analysis (TAT indicates tyrosine aminotransferase).

RC4-172/-147: GATGCGCTAGCCGACGAGGCAGGACGAA
GAGATGGCGCGCTACCCGCTGTTGAA
RC4-281/-256: AATTCGGCAGGAGCTAGCTCCACCGCTCCGCGCCTACTCTGTGAA
GATCCGCGACGACGCGAGGCGCCATGCGCGCGTGCGCGCCACTCTGCGA
RC4-187/-161: GATCCTGCTAGCCCGCATGCGCGCGCACCTGA
GACGATCGGGCGTACGCGCGCGTGGCTTCGGA
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 oligomers as described previously (14). Sequences were verified by the dideoxynucleotide sequencing method (20) after cloning the double-stranded oligomers into plasmids pGEM-Z7H(+) or pGEM-4B.

**Nuclear Extracts and Mobility Shift Assays**—Nuclear extracts were prepared from HeLa cells by an established method (21). For some experiments nuclear extracts were partially purified by heparin-Sepharose chromatography. The NRF-1 activity eluting from the column at 0.45 M KCl and precipitated with 50% ammonium sulfate was referred to as the HA45 fraction as previously described (14). Annealed oligonucleotides were 3'-end-labeled using Klenow enzyme and purified by gel electrophoresis. Binding reactions were performed in 25 μl of TM buffer (25 mM Tris, pH 7.9, 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM KC1, 10% (v/v) glycerol) containing between 0.02 and 4 pg of sonicated calf thymus DNA, and served as the template for the incorporation of 5-bromo-tyrosine aminotransferase -1090/-1069 was cloned into M13 phage DNA and purified by gel electrophoresis. Binding reactions were performed in 25 μl of TM buffer (25 mM Tris, pH 7.9, 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.1 M KCl). After incubation at room temperature for 15 min, the reaction mixture was irradiated for 30 min with a UV lamp at a distance of 2.5 cm. After addition of 50 μl of 2 X SDS sample buffer, mixtures were subjected to electrophoresis on a 10% polyacrylamide SDS gel and the radiolabeled DNA-protein complexes visualized by autoradiography of the dried gel. For competition experiments unlabeled competitor oligonucleotides were added at a 200-fold molar excess and incubated for 10 min prior to the addition of labeled probe.

**Cell Culture and DNA Transfection**—To assay the activity of transfected promoters containing the various NRF-1 sites, annealed oligomers were cloned as described previously (7, 14) into the polylinker of pCATB/Am (16, 17) containing the rat cytochrome c/CAT fusion gene deleted of cytochrome c sequences upstream from position -66. In these constructs, the cloned oligomers are positioned 95 base pairs upstream from the cytochrome c transcription start site. The plasmid p4xmMrp-311/-292, a derivative of pCATB/ZH(14), was constructed by converting the cloned oligonucleotide to a BglII site, followed by two rounds of dimerization using the flanking BamHI and BglII sites as described previously (14).

**Purification of NRF-1**—Nuclear extracts (21) were prepared from 12 liters of HeLa cells grown in spinner culture to a cell density of approximately 10⁶ cells/ml. Crude nuclear extract was applied to a heparin-Sepharose column (1.6 x 10 cm) equilibrated with buffer E containing 0.2 M KCl. Proteins were eluted as described for the first pass, the active fractions pooled, and subjected to a final purification step on a 3-ml NRF-1 affinity column equilibrated with the same buffer. The affinity column was prepared by ligating the double-stranded oligomer to a BglII site, followed by two rounds of chloramphenicol acetyltransferase assay, and one-half were used for the preparation of low molecular weight DNA by the Hirt method (24) to normalize for transfection efficiency. Promoter activities were the average of between two and five separate transfections of 3 plates each.

**UV-induced DNA Cross-linking**—Labeled DNA was prepared by the method of Chodosh et al. (23). The synthetic oligonucleotide tyrosine aminotransferase -1090/-1069 was cloned into M13 phage and served as the template for the incorporation of 5-bromo-2'deoxyuridine triphosphate (Pharmacia LKB Biotechnology Inc.) and [α-32P]dCTP. The DNA was digested with BamHI and HindIII and the 32-nucleotide probe gel-purified. Binding reactions were performed in 30-50 μl using the conditions described for nuclear shift assays except for the inclusion of 10 μg of BSA, 0.01-25 μg of sonicated calf thymus DNA, and 0.5-25 μg of denatured calf thymus DNA. NRF-1 activity was optimized for individual fractions. Approximately 0.5 units of NRF-1 binding activity (one unit is defined as the activity required to shift 5 fmol of labeled oligonucleotide tyrosine amino-transferase -1090/-1069 under standard mobility shift conditions) for fractions prior to the affinity steps and 1.0 unit of activity for affinity fractions were used. Following incubation at room temperature for 20 min, the reaction mixture was irradiated for 30 min with a UV lamp at a distance of 2.5 cm. After addition of 50 μl of 2 X SDS sample buffer, mixtures were subjected to electrophoresis on a 10% polyacrylamide SDS gel and the radiolabeled DNA-protein complexes visualized by autoradiography of the dried gel. For competition experiments unlabeled competitor oligonucleotides were added at a 200-fold molar excess and incubated for 10 min prior to the addition of labeled probe.
amidine gel. The region of the gel lane between 76 and 44 kDa was excised and cut into four slices. Proteins were eluted and renatured by the method of Briggs et al. (26) with slight modification. Briefly, gel slices were crushed with a siliconized glass pestle in siliconized 1.5-mL Eppendorf tubes. After addition of 1 ml of elution buffer containing 50 mM Tris-HC1 (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1% SDS, and 10 μg/μl BSA, each tube was rocked gently overnight at room temperature. The gel residue was removed by centrifugation and the protein precipitated from the supernatant by addition of 100 μg of BSA and five volumes of acetone. The resulting pellet was rinsed with 1 ml of 80% acetone, air-dried, and resuspended in 50 μl denaturation buffer containing 50 mM Tris-HC1 (pH 7.9), 12.5 mM MgCl2, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 μM ZnCl2, 50 mM KC1, 6 mM guanidine hydrochloride. After 20 min at room temperature, guanidine was removed by passage through a G-25 Sephadex (Sigma) column equilibrated with denaturation buffer without guanidine hydrochloride. NRF-1 activity was assessed by mobility shift as described above with the exception that binding reactions contained 3 μg of BSA and calf thymus DNA was omitted.

RESULTS

Seven Nuclear Genes Contain NRF-1 Recognition Sites—NRF-1 was initially defined by its ability to recognize a specific sequence contained within four recently isolated nuclear genes whose products were either directly or indirectly involved in mitochondrial respiratory function (7, 14). In two of these genes (cytochrome c and MRP RNA) the sites were found to be functionally conserved between rodent and human promoters. Fig. 1 shows a perfect match to the NRF-1 consensus at the 5' end of a recently published cDNA encoding γ-ATP synthase (15). In addition, strong similarities to the other NRF-1 sites were observed in genes encoding eIF-2α and tyrosine aminotransferase (Fig. 1). Both genes have sequences within their 5'-flanking DNA which differ by only a single nucleotide from the NRF-1 consensus derived from a compilation of functionally defined sites.

To determine whether the newly identified sequences were capable of specific NRF-1 recognition, synthetic oligomers of each were tested for their ability to displace NRF-1 binding to the rat cytochrome c (RC4) promoter site (Fig. 2). A crude nuclear extract prepared from HeLa cells generated a NRF-1-DNA complex (lane 1) that was unaffected by an excess of cloned oligomer containing the ATP transcription factor-binding site from RC4 (RC4 –281/–256, lane 2) but was completely inhibited by an excess of unlabeled RC4 NRF-1 oligomer (RC4 –172/–147, lane 3). Likewise, NRF-1 complex was strongly inhibited by an excess of NRF-1 oligomers from γ-ATP synthase +1/+20 (lane 4), eIF-2α –41/–21 (lane 5) or tyrosine aminotransferase –1090/–1069 (lane 6). Under identical conditions the same NRF-1 oligomers from the various genes had no effect upon the multiple shifted complexes formed with the RC4 –281/–256 ATF site (Fig. 2, lanes 16–21) confirming that the inhibition was NRF-1 specific. In addition, labeled NRF-1 oligomers from γ-ATP synthase (lane 7), eIF-2α (lane 10), and tyrosine aminotransferase (lane 13) genes formed shifted complexes of identical mobility to that formed with the RC4 site (lane 1). These complexes were unaffected by an excess of unlabeled RC4 ATF oligomer (lanes 8, 11, and 14) but were competed away by an excess of RC4 NRF-1 oligomer (lanes 9, 12, and 15). These results strongly suggest that the shifted complexes formed with each of the sites requires a specific interaction with NRF-1.

NRF-1 Complexes Formed with the Various Target Sequences Are Indistinguishable—If specific recognition of each gene occurs through the same molecular species, the various sites should display similar patterns of nucleotide contacts. Methylation interference was performed to identify the major groove guanine nucleotides involved in NRF-1 binding to γ-ATP synthase +1/+20, eIF-2α –42/–21 and tyrosine aminotransferase –1090/–1069. As shown in Fig. 3A, methylation of similar nucleotides within the core recognition sequence from each gene either completely or partially interfered with the formation of a specific DNA-protein complex. All of the nucleotide contacts within the three new sequences are found within the consensus of contact nucleotides derived from a comparison of the four previously characterized NRF-1 binding sites (14) and coincide with the directly repeated TGGCGA motifs (Fig. 3B).

UV-induced DNA-protein cross-linking was performed to investigate the size and complexity of the proteins responsible for the NRF-1 binding activity present in crude nuclear extracts. Bromodeoxyuridine was incorporated in place of thymidine into the NRF-1 site from the tyrosine aminotransferase gene that was also radiolaabeled with [α-32P]dCTP. This site was chosen because it contains an additional A/T base pair for bromodeoxyuridine incorporation which would facilitate UV cross-linking efficiency. Two labeled DNA-protein complexes, represented by a major band at about 90 kDa and a less intense band at about 110 kDa, were detected following separation of UV-irradiated complexes formed with fraction HA45 on denaturing acrylamide gels (Fig. 4, lane 1). Assuming a molecular mass of about 20 kDa for the DNA probe these corresponded to polypeptides of about 70 and 90 kDa for the

### Table: Summary of NRF-1 recognition sites

| Gene Name | Abbreviated Name | Sequence |
|-----------|-----------------|----------|
| Rat somatic cytochrome c | RCS | -173 stgcctag CcGCGATGCGCG gcgcctt -147 |
| Human somatic cytochrome c | HCS | +16 stgcctag CcGCGATGCGCG gcgcctt -150 |
| Rat cytochrome c oxidase Vic-2 | COX-Vic-2 | +46 stgcctag CcGCGATGCGCG gcgcctt -20 |
| Human ubiquinone-binding protein | hQF | -46 ggcccgt CcGCGATGCGCG gcgcctt -75 |
| Mouse MRP RNA | mMRP RNA | -313 ggccgct CcGCGATGCGCG gcgcctt -289 |
| Human MRP RNA | hMRP RNA | -299 ggcgcct CcGCGATGCGCG gcgcctt -273 |
| Bovine ATP synthase γ-subunit | γ-ATPS | +1 ccc CcGCGATGCGCG gtgag -20 |
| Human eukaryotic initiation factor 2 γ-subunit | eIF-2α | -42 ggcccgt CcGCGATGCGCG gcgcctt -21 |
| Rat tyrosine aminotransferase | TAT | -1090 ggcccgt CcGCGATGCGCG gcgcctt -1069 |

**Fig. 1.** Summary of NRF-1 recognition sites. Previously identified functional NRF-1 sites (14) are compared with the newly identified NRF-1 sequences found in the γ-ATP synthase (γ-ATPS) (16), eIF-2α (16), and tyrosine aminotransferase (TAT) (17) genes. Numbering is from the beginning of the cDNA for γ-ATP synthase or from the known transcription initiation sites for eIF-2α and tyrosine aminotransferase genes. *Uppercase letters* denote matches to the NRF-1 consensus binding site derived from a comparison of all of the sequences and shown in the bottom line.
major and minor species, respectively. Neither complex was formed when cross-linking was performed in the presence of an excess of unlabeled NRF-1 oligomers from the functional recognition sites of each of the seven target genes (lanes 2–8). However, both complexes were formed in the presence of an excess of an unrelated RC4 oligomer (lane 11) and an hCC1 oligomer (lane 10). The latter was derived from a region of the human cytochrome c1 gene which closely resembles the NRF-1 consensus sequence yet has been found to have no NRF-1 binding or transcriptional activity (14). The hCC1 oligomer has two nucleotide differences with hCC1 and has been shown to be active in both NRF-1 binding and transcriptional activity (14). An excess of unlabeled hCC1 abolished the formation of both labeled complexes (lanes 9), further confirming their specificity.

NRF-1 Recognition Sites from γ-ATP Synthase, eIF-2α, and Tyrosine Aminotransferase Mediate Transcriptional Activation in Transfected Cells—The previously identified NRF-1 sequences from respiratory genes were all able to stimulate transcription when cloned upstream in an RC4 promoter/CAT fusion gene (14). The expression vector RC4CATBA.I utilized for transient transfections, contains the RC4 promoter activity. When vectors containing NRF-1 sites were transfected into COS-1 cells using pGEM-4 blue as a nonspecific carrier DNA, the level of promoter stimulation directed by γ-ATP synthase +1/+20, eIF-2α –42/–21, and tyrosine aminotransferase –1090/–1069 oligomeric complexes were partially methylation and subjected to preparative scale mobility shift analysis using HeLa extract fraction HA45 as described under “Experimental Procedures.” F and B indicate the piperidine cleavage products of DNA fragments 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. Filled circles denote guanosine bases that when methylated strongly inhibited NRF-1 binding; open circles denote guanosines where methylation partially interfered with NRF-1 binding. Open circles denote guanosines where methylation partially interfered with NRF-1 binding. B, summary of the guanosine nucleotide contacts made on both the upper and lower strands of each newly identified NRF-1 site compared with the previously established NRF-1 binding site consensus sequence. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.

Fig. 2. Binding specificities of DNA-protein complexes formed with NRF-1. End-labeled oligonucleotides from RC4 –172/–147 (lanes 1–6), γ-ATP synthase +1/+20 (lanes 7–9), eIF-2α –42/–21 (lanes 10–12), tyrosine aminotransferase –1090/–1069 (lanes 13–15), and RC4 –281/–256 (lanes 15–20) were incubated with 25 µg of HeLa 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 the unlabeled competitor oligonucleotide indicated above each lane. None refers to no competitor. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.

Fig. 3. Methylation interference analysis of NRF-1 binding. A, end-labeled DNA fragments containing the cloned γ-ATP synthase +1/+20, eIF-2α –42/–21, and tyrosine aminotransferase –1090/–1069 oligonucleotides were partially methylated and subjected to preparative scale mobility shift analysis using HeLa extract fraction HA45 as described under “Experimental Procedures.” F and B indicate the piperidine cleavage products of DNA fragments 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. Filled circles denote guanosine bases that when methylated strongly inhibited NRF-1 binding; open circles denote guanosines where methylation partially interfered with NRF-1 binding. B, summary of the guanosine nucleotide contacts made on both the upper and lower strands of each newly identified NRF-1 site compared with the previously established NRF-1 binding site consensus sequence. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.

Fig. 4. Specific NRF-1 complexes detected by UV-induced DNA-protein cross-linking. Aliquots of the heparin-agarose 0.45 M wash containing approximately 3 units of NRF-1 activity were incubated with radiolabeled tyrosine aminotransferase –1090/–1069 containing bromodeoxyuridine. Samples were irradiated under UV light prior to separation on a 10% SDS-polyacrylamide gel. Competition assays were performed using a 200-fold molar excess of unlabeled competitor oligonucleotide as indicated above each lane. None indicates no competitor added. Standards of the indicated molecular masses are indicated at the left. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.
The activity produced by the parent promoter vector with no cloned were normalized for transfection efficiency by Hirt DNA analysis. Chloramphenicol acetyltransferase (CAT) activities oligonucleotide was defined as 1.0 for each carrier DNA. using known amounts of BSA as a standard.

The stimulation of CAT activity by RC4-187/-161, a functionally independent cytochrome c promoter element distinct from the NRF-1 site (7), was unaffected by the excess of unlabeled RC4-172/-147 (specific) or RC4-281/-256 (nonspecific) competitor oligonucleotide as indicated above each lane. None indicates no competitor added. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.

excess) of NRF-1 sites was introduced in trans by using an excess of carrier plasmid containing four tandem copies of the mMRP RNA-311/-292 NRF-1 oligomer (p4xMRP-311/-292). Cotransfection with this plasmid resulted in a substantial reduction in the CAT activity directed by each of the cloned NRF-1 oligomers (Table I). The reduction of promoter activity was the result of specific trans-competition because the stimulation of CAT activity by RC4-187/-161, a functionally independent cytochrome c promoter element distinct from the NRF-1 site (7), was unaffected by the excess of cotransfected NRF-1 sites. These results demonstrate that the promoter activation mediated by the three newly identified NRF-1 sites occurs through a specific interaction of NRF-1 with its promoter recognition sequence.

Purification of NRF-1 to Near Homogeneity and Its Specific Recognition of Target Genes—Definitive proof that the same NRF-1 polypeptide is responsible for specific recognition of the various target genes requires the purification of NRF-1. To this end, NRF-1 was purified approximately 33,000-fold from a HeLa cell nuclear extract (Table II, Fig. 5A). Following heparin-agarose and DNA-cellulose chromatography, the partially purified material was subjected to three passages through a specific DNA affinity column constructed by coupling a concatenated oligomer from the COX-Vic-2 NRF-1 recognition site to cyagen bromide-activated Sepharose.

![Fig. 5. Specific target site recognition by NRF-1 purified over 33,000-fold.](image-url)

### Table 1

| Cloned oligonucleotide | Relative CAT activitya |
|------------------------|------------------------|
|                        | pGEM-4 blue | p4xMRP RNA-311/-292 |
| None                   | 1.0       | 1.0                  |
| RC4-172/-147           | 5.1       | 1.9                  |
| COX-Vic-2+20/+46       | 31.7      | 4.1                  |
| γ-ATPs +1/+20          | 9.6       | 2.6                  |
| eIF-2α -42/-21         | 7.5       | 1.7                  |
| TAT -1090/-1069        | 28.6      | 3.8                  |
| RC4-187/-161           | 44.0      | 5.52                 |

* The indicated oligonucleotides were cloned upstream of the cytochrome c gene truncated at position -66 in the vector pRC4CATBΔ/-66BΔ.

0.5 μg of the promoter vectors were transfected into COS-1 cells using 20 μg of either pGEM-4 blue or p4xMRP RNA-311/-292 as carrier DNA. Chloramphenicol acetyltransferase (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.

### Table 2

| Fraction               | Protein concentration | Total activity | Specific activity | Purification | Yield |
|------------------------|-----------------------|----------------|------------------|--------------|-------|
| Nuclear extract        | 241 mg                | 1,448 units    | 0.006            | 100%         |       |
| Heparin-agarose        | 38 mg                 | 1,050 units    | 0.028            | 72.3%        |       |
| DNA-cellulose          | 18 mg                 | 573 units      | 0.032            | 39.6%        |       |
| DNA affinity           |                       |                |                  |              |       |
| P1                     | 0.72                  | 541 units      | 0.77             | 128%         | 37.3% |
| PII                    | 0.035                 | 1,134 units    | 32.4             | 5,400%       | 78.3% |
| PIII                   | 0.001                 | 200 units      | 0.018            | 33,000%      | 13.8% |

* Purification of NRF-1

* Protein concentration was determined by the Bradford assay in fractions prior to the first affinity step. For second and third pass affinity fractions, protein concentrations were estimated by densitometric scanning of silver-stained 10% SDS-polyacrylamide gel lanes using known amounts of BSA as a standard.

* One unit is defined as the activity required to shift 5 fmol of labeled oligonucleotide tyrosine aminotransferase-1090/-1069 under standard mobility shift conditions.

* P1, first pass affinity fraction; PII, second pass affinity fraction; PIII, third pass affinity fraction.

### Legend

- **A**: Stimulation of promoter activity by cloned oligonucleotides
  - **B**: Purification of NRF-1

- **STD**: Standard
- **NE**: Nuclear extract
- **HA**: Heparin-agarose
- **DC**: DNA-cellulose
- **P1**: First pass affinity fraction
- **P2**: Second pass affinity fraction
- **P3**: Third pass affinity fraction

- **Fig. 5A**: Lane 7.

- **Fig. 5B**: Lane 1.

- **Fig. 5C**: Lane 2.

- **Fig. 5D**: Lane 3.

- **Fig. 5E**: Lane 4.

- **Fig. 5F**: Lane 5.

- **Fig. 5G**: Lane 6.

- **Fig. 5H**: Lane 7.

- **Fig. 5I**: Lane 8.

- **Fig. 5J**: Lane 9.

- **Fig. 5K**: Lane 10.

- **Fig. 5L**: Lane 11.

- **Fig. 5M**: Lane 12.

- **Fig. 5N**: Lane 13.

- **Fig. 5O**: Lane 14.

- **Fig. 5P**: Lane 15.
with the latter as a labeled probe (lanes 13–15). These results demonstrate that the NRF-1 activities responsible for specific recognition of the various target genes can be purified approximately 33,000-fold and likely reside in one or both of the major protein bands visualized by silver staining.

If the same proteins are responsible for specific binding to the various target genes the same DNA-protein complexes should be detectable by UV-induced DNA cross-linking using fractions from throughout the purification scheme. As shown in Fig. 6A, the same major DNA-protein complexes of 90 and 110 kDa detected using crude extracts were also found using NRF-1-containing fractions from each of the chromatographic steps including the most highly purified affinity fractions (Fig. 6A, lane 6). Like the complexes formed using the HA45 fraction (Fig. 4) those formed using affinity-purified fractions (Fig. 6B, lane 1) were specifically competed away by an excess of unlabeled NRF-1 oligomers from RC4 –172/-147 (lane 2), eIF-2a -42/-21 (lane 3), γ-ATP synthase +1/+20 (lane 4), and tyrosine aminotransferase –1090/-1069 (lane 5) but not by an excess of RC4 –281/-256 ATF oligomer (lane 6). When corrected for the labeled oligomer, the 90-kDa complex corresponds to a protein of about 70 kDa. This agrees well with the major polypeptide of 68 kDa detected in the most highly purified affinity fraction, strongly suggesting that it is the NRF-1 protein responsible for recognizing the various target genes (Fig. 6A, lane 6).

The presence of a 110-kDa cross-linked complex using the most highly purified affinity fraction was unexpected. This complex implied the existence of a 90-kDa NRF-1 species assuming the complex resulted from an equimolar ratio of protein and labeled oligomer. No protein of 90 kDa was visible in the affinity-purified NRF-1 fraction, nor could a NRF-1 binding activity be detected in this region of the gel lane upon renaturation (see below). Moreover, when the UV-cross-linked material was subjected to nuclease digestion, only a single specific band corresponding to a complex of about 76 kDa was detected upon denaturing gel electrophoresis (Fig. 6C, lanes 2–4). Allowing for the 17 nucleotides known to be protected from nuclease digestion by NRF-1 binding (7), this band corresponded to a protein of about 66 kDa. Once again this agrees well with the major 68-kDa band detected upon denaturing gel electrophoresis of the most highly purified NRF-1 fraction. The elimination of the larger specific complex upon nuclease digestion indicates that it is comprised of additional nucleic acid rather than a larger polypeptide. One possibility is that the 110-kDa cross-linked complex results from the binding of NRF-1 to dimerized probe which would account for the extra 20 kDa of nuclease-sensitive material. Nevertheless, the only specific, nuclease-resistant complex corresponds to a polypeptide whose estimated mass agrees with that of the major affinity-purified protein.

To further establish that the 68-kDa protein is the active species, slices spanning the range between 76 and 44 kDa were excised from a denaturing acrylamide gel (indicated in Fig. 5A), and the proteins eluted, renatured, and assayed for NRF-1 binding activity. Of the four slices taken from the region of the gel lane containing the protein bands visible by silver staining, only slice 1, corresponding to the 68-kDa protein, displayed specific NRF-1 binding activity (Fig. 7A, lanes 3 and 4). The mass of this protein is in good agreement with the major 70-kDa cross-linked polypeptide. Further slices in the direction of higher molecular mass failed to reveal an activity that would account for the larger UV-cross-linked complex (not shown). The DNA-protein complex formed with the renatured 68-kDa protein had a mobility identical to that formed with affinity purified NRF-1 (compare lanes 1 and 3).

**FIG. 6.** Copurification and target site specificity of NRF-1 complexes detected by UV-induced DNA-protein cross-linking. A, UV cross-linking was performed using NRF-1-containing protein fractions from the various purification steps as depicted in Fig. 5A. Reactions contained approximately 0.5 units of NRF-1 activity from fractions prior to the affinity step and approximately 1 unit of activity from the affinity-purified fractions. Radiolabeled, bromodeoxyuridine-substituted tyrosine aminotransferase –1090/-1069 was incubated with crude nuclear extract (NE, lane 1), heparin-agarose 0.45 M wash (HA, lane 2), DNA-cellulose fraction (DC, lane 3), first pass affinity fraction (PI, lane 4), second pass affinity fraction (PII, lane 5), third pass affinity fraction (PIII, lane 6). Complexes were formed by UV irradiation and separated on a 10% SDS-polyacrylamide gel. Standards of known molecular mass are indicated at the left. B, specificity of the UV-induced DNA-protein complex formed with third pass affinity material as described in panel A was assessed by including a 200-fold molar excess of unlabeled competitor oligonucleotides from the various target sites (lanes 2–5) as indicated above each lane. Complexes were separated on a 10% SDS-polyacrylamide gel after UV irradiation. None (lane 1) indicates no competitor, while RC4 –281/-256 (lane 6) serves as a nonspecific competitor. Standards of known molecular mass are indicated at the left. C, nuclease digestion of the NRF-1-DNA complex. Prior to separation on a 10% SDS-polyacrylamide gel, complexes formed with affinity-purified NRF-1 (1 unit of third pass affinity fraction) were either untreated (lane 1) or treated with 6 units of DNase I and 9 units of micrococcal nuclease in the presence of 10 mM CaCl2 and MgCl2 (lanes 2–4). Complex formation was determined in the absence of competitor (lanes 1 and 2) and in the presence of a 200-fold molar excess of unlabeled specific (RC4 –172/-147, lane 3) or nonspecific (RC4 –281/-256, lane 4) competitor oligonucleotide as indicated above each lane. Standards of known molecular mass are indicated at the left. γ-ATPS, γ-ATP synthase; TAT, tyrosine aminotransferase.
purified NRF-1. Following elution and renaturation the protein re-
specific (RC4 -172/-147) or nonspecific (RC4 -281/-256) end-
present  in each of the  target genes.

binding reactions had no effect on  either  the mobility or
competitor with end-labeled oligonucleotide RC4 -172/-147 in the absence of
the unlabeled competitor oligonucleotides indicated above each lane
recovered from a denaturing polyacrylamide gel slice.
slices spanning the region from 76 to 44 kDa as indicated in Fig.
NRF-1. Competition binding  assays demonstrated  that  the
intense  protein bands  are  contaminants  and  not  subunits of
shown),  indicating that  the 55-kDa protein and  other less
formed using the second pass
7B, lane 2) was displaced using an excess of unlabeled NRF-
eIF-2a -42/-21
A, eIF-2a -42/-21
ATP synthase; TAT, tyrosine aminotransferase.
 Addition of renatured material from gel slices 2 through 4 to
binding reactions had no effect on either the mobility or
intensity of the complex formed using slice 1 protein (not
shown), indicating that the 55-kDa protein and other less
intense protein bands are contaminants and not subunits of
NRF-1. Competition binding assays demonstrate that the
collided with the renatured material from slice 1 (Fig.
lanes 1 and 2 depict complexes formed with the second pass affinity
fraction (PII). B, renatured material from gel slice 1 was incubated with end-labeled oligonucleotide RC4 -172/-147 in the absence of competitor (lane 2) or in the presence of a 200-fold molar excess of
the unlabeled competitor oligonucleotides indicated above each lane
(lanes 3-7). For comparison lane 1 shows the NRF-1-DNA complex
formed using the second pass (PII) affinity fraction. γ-ATPS, γ-ATP
synthase; TAT, tyrosine aminotransferase.

DISCUSSION

The limited coding capacity of mammalian mitochondrial
DNA necessitates a major contribution by the nuclear genome
to the control of mitochondrial function. We have investigated
the structure and expression of mammalian cytochrome c
genes with the aim of identifying control elements common
to nucleus-encoded respiratory genes. As a result of this
approach, NRF-1 was discovered as a trans-activator of cyto-

chome c gene expression (7) and its recognition site found
ports  the role of NRF-1 in respiratory  chain expression. While
this work was in progress the sequence of the mouse gene
coding cytochrome c oxidase subunit Vb was reported (27).
Inspection of the 5'-flanking sequence of this gene reveals a
near perfect match to the NRF-1 consensus binding site (−92
CCGACATGGCCA−103) located upstream from the site of
transcription initiation. It should be noted that at least three
other respiratory genes encoding cytochrome c1 (28), cyto-

chrome oxidase subunit IV (29), and the ATP synthase β-
subunit (30) appear to be devoid of NRF-1 recognition sites.
However, it is intriguing that two of these genes encoding
cytochrome oxidase subunit IV and the ATP synthase β-
subunit have binding sites for a second factor designated as
NRF-2 (31), suggesting that subsets of respiratory chain genes
may be differentially regulated through the utilization of
multiple NRF factors. Alternatively, the present finding of a
NRF-1 site in an upstream enhancer of the tyrosine amino-
transferase gene raises the possibility that NRF-1 activation
sites have not yet been detected in some respiratory chain
genes because they reside in unknown upstream or down-
stream enhancer elements.

The demonstration of functional NRF-1 activation sites in
either eIF-2α and tyrosine aminotransferase genes indicates
that transcriptional control by NRF-1 is not exclusive to
genes directly involved in mitochondrial function. This
finding supports a more integrative role for NRF-1 in metabolic
regulation. In both cases the NRF-1 sequences coincide with
regions of the promoter identified by their hypersensitivity to
DNase I digestion (17, 32). The NRF-1 site in the tyrosine
aminotransferase gene corresponds to a footprint obtained in
vivo using dimethyl sulfate (32). Although the tyrosine ami-
notransferase gene is known to be highly regulated, this and
Nucleus Cytoplasm Mitochondria

FIG. 8. Summary of the interactions between NRF-1 and
seven target genes. Arrows depict the potential activation of the
indicated target genes by NRF-1 and the site of action of the various
gene products on mitochondrial function. ETC, the electron transport
chain and oxidative phosphorylation system of the mitochondrial
inner membrane.
adjacent footprints occur in the DNase I hypersensitive region of an upstream enhancer for which no regulatory function has yet been assigned. The NRF-1 site in the eIF-2α gene corresponds to a palindromic sequence which binds a protein in the range of 66–68 kDa (32). This protein appears to stimulate transcription through two nonidentical binding sites in the eIF-2α promoter. Although only one of the sites is tested here, the other (–54 TGCACCAGCGAG) is thought to be a lower affinity site, also contains a single mismatch to the NRF-1 consensus and is almost certainly a recognition site for NRF-1. Although it is likely that the same 68-kDa protein is responsible for the recognition of these as well as the other NRF-1 sites, the possibility that structurally distinct proteins are involved in the recognition of the various target genes cannot be excluded. Families of transcriptional activators whose members are similar in size and bind identical target sequences have been described (33).

If NRF-1 participates in respiratory chain expression what is the significance of finding NRF-1 activation sites in non-respiratory genes? A feature common to both eIF-2α and tyrosine aminotransferase is that they are both involved in the rate-limiting steps of their respective pathways. Translation factor eIF-2 catalyzes the GTP-dependent interaction of the 40S ribosomal subunit with the initiator tRNA and is known to be an important site for regulation of translation initiation (18). Because protein synthesis is an energy-expensive process it may be useful, under certain physiological conditions, to coordinate the synthesis of initiation factors with respiratory chain proteins.

Tyrosine aminotransferase is a highly regulated, liver-specific enzyme that initiates the degradation of tyrosine ultimately leading to the production of fumarate and acetoacetate (reviewed in Ref. 19). Fumarate enters the citric acid cycle where it gives rise to reducing equivalents which can feed directly into the respiratory chain. Acetoacetate, while produced in the liver, is transported to other tissues such as heart muscle, where it serves as a preferred substrate for oxidative energy production (34). NRF-1 may provide a regulatory connection between tyrosine catabolism and mitochondrial respiration for the generation of respiratory fuels. It remains to be determined whether NRF-1 activation sites will appear in other genes encoding proteins involved in the rate-limiting steps of other key biosynthetic and degradative pathways.

Acknowledgments—We thank J. Virbasius for critical comments and the Northwestern University Biotechnology Facilities for synthesis of oligonucleotides.

REFERENCES
1. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 269–333
2. Nagley, P. (1991) Trends Genet. 7, 1–4
3. Scarpulla, R. C. (1984) Mol. Cell. Biol. 4, 2279–2298
4. Virbasius, J. V., and Scarpulla, R. C. (1988) J. Biol. Chem. 263, 6791–6796
5. Evans, M. J., and Scarpulla, R. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9625–9629
6. Evans, M. J., and Scarpulla, R. C. (1988) Mol. Cell. Biol. 8, 35–41
7. Evans, M. J., and Scarpulla, R. C. (1989) J. Biol. Chem. 264, 14361–14368
8. Ip, Y. T., Poon, D., Stone, D., Granner, D. K., and Chalkley, R. (1990) Mol. Cell. Biol. 10, 3770–3781
9. Suzuki, H., Hosokawa, Y., Toda, R., Nishikimi, M., and Ozawa, T. (1989) Biochem. Biophys. Res. Commun. 161, 371–376
10. Suske, G., Enders, C., Schlerf, A., and Kadenbach, B. (1988) DNA 7, 163–171
11. Chang, D. D., and Clayton, D. A. (1987) EMBO J. 6, 409–417
12. Chang, D. D., and Clayton, D. A. (1989) Cell 66, 131–139
13. Topper, J. N., and Clayton, D. A. (1990) Nucleic Acids Res. 18, 793–799
14. Evans, M. J., and Scarpulla, R. C. (1990) Genes Dev. 4, 1023–1034
15. Dyer, M. R., Gay, N. J., Powell, S. J., and Walker, J. E. (1989) Biochemistry 28, 3673–3680
16. Hummelin, M., Säfer, B., Chiorini, J. A., Hershey, J. W. B., and Cohen, R. B. (1989) Gene (Amst.) 81, 315–324
17. Becker, P. B., Ruppert, S., and Schütz, G. (1987) Cell 51, 435–443
18. Hershey, J. W. B. (1991) Annu. Rev. Biochem. 60, 717–755
19. Hargrove, J. L., and Granner, D. K. (1985) in Transaminases (Christen, P., and Metzger, P. E., eds) pp. 511–532, John Wiley & Sons, Inc., New York
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
21. Dignam, J. D., Lebowitz, R. M., and Roeder, G. R. (1983) Nucleic Acids Res. 11, 1475–1489
22. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
23. Chodosh, L. A., Carthew, R. W., and Sharp, P. A. (1986) Mol. Cell. Biol. 6, 4725–4733
24. Hirt, B. (1987) J. Mol. Biol. 26, 365–369
25. Kadonaga, J. T., and Tjian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5889–5893
26. Briggs, M. R., Kadonaga, J. T., Beil, S. P., and Tjian, R. (1986) Science 234, 47–52
27. Basu, A., and Avadhani, N. G. (1991) J. Biol. Chem. 266, 15450–15456
28. Suzuki, H., Hosokawa, Y., Nishikimi, M., and Ozawa, T. (1989) J. Biol. Chem. 264, 1366–1374
29. Virbasius, J. V., and Scarpulla, R. C. (1990) Nucleic Acids Res. 19, 6581–6586
30. Ohta, S., Tomura, H., Matsuda, K., and Kagawa, Y. (1988) J. Biol. Chem. 263, 11257–11262
31. Virbasius, J. V., and Scarpulla, R. C. (1991) Mol. Cell. Biol. 11, 5631–5638
32. Jacob, W. F., Silverman, T. A., Cohen, R. B., and Safer, B. (1989) J. Biol. Chem. 264, 20372–20384
33. Hai, T., Liu, F., Coukos, W. J., and Green, M. R. (1989) Genes Dev. 3, 2083–2090
34. Stryer, L. (1988) Biochemistry, p. 479, W. H. Freeman and Co., New York