NRF-1, an activator involved in nuclear–mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators

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Nuclear respiratory factor 1 (NRF-1) was first discovered as an activator of the cytochrome c gene and was subsequently found to play a broader role in nuclear–mitochondrial interactions. We have now cloned a HeLa cDNA encoding NRF-1 using degenerate oligomers derived from tryptic peptide sequences for PCR amplification. The cDNA-encoded protein was indistinguishable from the authentic HeLa cell factor on denaturing gels, displayed the expected NRF-1 DNA-binding specificity, and made the same guanine nucleotide contacts as HeLa NRF-1 on binding known NRF-1 recognition sites. Antiserum raised against the highly purified recombinant protein recognized the identical DNA–protein complex formed using either a crude nuclear fraction or nearly homogeneous HeLa NRF-1. Recombinant NRF-1 also activated transcription through specific sites from several NRF-1-responsive promoters, confirming both the transcriptional activity and specificity of the cDNA product. Portions of NRF-1 are closely related to sea urchin P3A2 and the erect wing (EWG) protein of Drosophila. Both are recently identified developmental regulatory factors. The region of highest sequence identity with P3A2 and EWG was in the amino-terminal half of the molecule, which was found by deletion mapping to contain the DNA-binding domain, whereas the carboxy-terminal half of NRF-1 was highly divergent from both proteins. The DNA-binding domain in these molecules is unrelated to motifs found commonly in DNA-binding proteins; thus, NRF-1, P3A2, and EWG represent the founding members of a new class of highly conserved sequence-specific regulatory factors.

[Key Words: Oxidative phosphorylation; nuclear respiratory factors; mitochondria; transcription]

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The vertebrate mitochondrion contains its own genome, along with the machinery required for its autonomous transcription, translation, and replication (Attardi and Schatz 1988; Clayton 1991; Wallace 1992). Mitochondrial DNA, however, has a coding capacity limited to only 13 respiratory chain polypeptides, and 2 ribosomal and 22 transfer RNAs. Most respiratory proteins and all of those necessary for maintenance and expression of the mitochondrial genome are encoded in the nuclear DNA. Thus, understanding the genetic control of mitochondrial function largely becomes a problem of identifying the nuclear genes involved and investigating potential mechanisms of regulated expression.

Investigations of nuclear–mitochondrial interactions in mammalian cells have led to the cloning of nuclear gene products required for mitochondrial DNA transcription and replication (Clayton 1991). The human and mouse genes for the RNA subunit of MRP endonuclease, a ribonucleoprotein enzyme that is thought to cleave light-strand transcripts to form primers for heavy-strand DNA replication, have been cloned (Chang and Clayton 1989; Topper and Clayton 1990). A second nuclear gene product linking transcription and replication is mitochondrial transcription factor 1 (mtTF1), now called mtTFA (Xu and Clayton 1992). This factor recognizes the divergent heavy- and light-strand promoters to stimulate transcription initiation by mitochondrial RNA polymerase in vitro (Fisher et al. 1987). Because light-strand transcripts cleaved by mitochondrial RNA processing (MRP) endonuclease prime heavy-strand replication, mtTFA has the potential to modulate both transcription and replication of mitochondrial DNA (Clayton 1991). The importance of mtTFA to mitochondrial function in vivo is supported by the observation that a null mutation in the yeast counterpart to mtTFA (ABF2) results in the loss of mitochondrial DNA (Diffley and Stillman 1991). Interestingly, this phenotype can be rescued by expression of human mtTFA in yeast (Paris et al. 1993). Regulatory elements common to nuclear genes with
products that function in the mitochondria have also been described [Nagley 1991, Wallace 1993]. Analysis of cytochrome c and cytochrome oxidase promoters has led to the identification of transcriptional activators designated as nuclear respiratory factors (NRF)-1 (Evans and Scarpulla 1989, 1990; Chau et al. 1992) and -2 (Virbasius and Scarpulla 1991; Virbasius et al. 1993). Functional NRF-1 sites have been found in genes encoding cytochrome c and at least one subunit each of respiratory complexes III, IV, and V (Evans and Scarpulla 1989, 1990; Chau et al. 1992), suggesting a role for the factor in the coordinate expression of respiratory chain subunits. NRF-1 may also participate in mitochondrial gene expression through its sequence-specific activation of genes encoding both the MRP RNA (Evans and Scarpulla 1990) and mtTFA (this paper, Virbasius and Scarpulla 1994). Activity of the proximal mtTFA promoter is highly dependent on NRF-1 in both transfected cells and in in vitro transcription assays (Virbasius and Scarpulla 1994). Similarly, expression of the gene encoding 5-aminolevulinate (5-ALA) synthase, the rate-limiting enzyme in the biosynthesis of heme for respiratory cytochromes, requires two NRF-1 recognition sites within its promoter region (Braidotti et al. 1993). These findings are consistent with an integrative role for NRF-1 in controlling nuclear–mitochondrial interactions in higher organisms. In addition, functional NRF-1-binding sites are present in the genes for tyrosine aminotransferase and the translation initiation factor eIF-2a (Chau et al. 1992). Like 5-ALA synthase, these two proteins participate in the rate-limiting steps of their respective pathways of tyrosine catabolism and protein synthesis. Thus, NRF-1 may integrate a number of metabolic processes by regulating the genes encoding key enzymes.

As a prelude to molecular cloning, NRF-1-binding activity was purified over 30,000-fold to near homogeneity and was found to reside in a single polypeptide of 68 kD (Chau et al. 1992). Here, we use the sequences of tryptic peptides to obtain a cDNA clone that encodes a protein with the DNA-binding and transcriptional specificities expected for NRF-1. Deletion mapping of NRF-1 establishes that its DNA-binding domain coincides with a region of high sequence similarity with P3A2 [Calzone et al. 1991; Hoog et al. 1991] and erect wing (EWG) [Desimone and White 1993], two recently identified developmental regulatory factors. Thus, these proteins define a new family of sequence-specific regulators that share a conserved DNA-binding domain. On the basis of functional analysis of over a dozen NRF-1 sites, we predict that the NRF-1 DNA-binding domain participates in the expression of >50 mammalian genes of known sequence.

**Results**

*Molecular cloning and overexpression of recombinant NRF-1*

Tryptic peptides were derived from ~50 pmoles of purified HeLa NRF-1 [Chau et al. 1992]. Two peptide peaks from HPLC chromatography were chosen for microsequencing. NRF-1 [72] yielded 26 residues (SMILEDLE-SALAEHAPQEVNSEL) from a single homogenous peptide, whereas NRF-1 [38] was a mixture of two peptides with a novel 8-residue sequence (VSWTQALR) derived from the secondary product. The major NRF-1 [38] peptide was from Ku antigen, a known contaminant of DNA-binding proteins [Kadonaga 1991]. A series of degenerate oligonucleotides was designed for PCR amplification of total cDNA prepared from HeLa poly(A)+ RNA. Two of these probes yielded a 269-nucleotide PCR product containing an open reading frame that was subsequently used as a probe to obtain a 3-kb HeLa cDNA clone. The putative NRF-1 cDNA had a 503-amino-acid open reading frame containing the complete sequence of the PCR product flanked by the two NRF-1 peptides [Fig. 1].

The cDNA product was overexpressed in Escherichia coli using an inducible T7 expression system [Studier et al. 1990]. A transformant containing the NRF-1-coding region transcribed from an inducible T7 promoter gave a major 68-kD protein on induction [Fig. 2, lanes 2,3]. The 68-kD mass of the induced protein is identical to that determined previously for highly purified HeLa NRF-1 [Chau et al. 1992] but is greater than the 54-kD mass predicted by the NRF-1 open reading frame. As only the open reading frame fragment was cloned into the expression vector, the mass of NRF-1 appears to be overestimated on denaturing gels. The induced 68-kD protein was purified to >95% purity from sonified extracts by ammonium sulfate precipitation [lane 4] and heparin-agarose fractionation [lane 5].

To determine whether the cDNA-encoded recombinant protein was the same as that present in DNA–protein complexes formed using HeLa cell NRF-1, goat antiserum was raised against the purified recombinant protein and tested for its ability to "supershift" NRF-1–DNA complexes in a gel-retardation assay. DNA–protein complexes of identical migration were formed with a labeled rat cytochrome c NRF-1 oligomer (RC4-172/-147) using crude nuclear extract [Fig. 3, lane 1], affinity-purified NRF-1 [lane 4], or recombinant NRF-1 [lane 7]. In each case, the complex was supershifted with antiserum raised against recombinant NRF-1 [lanes 3, 6, 9] under conditions where preimmune serum had no effect [lanes 2, 5, 8]. Variations in the antibody complexes formed with affinity-purified and recombinant proteins result from differences in the antigen–antibody ratios present in each reaction. The multiple complexes observed previously with binding of NRF-2 to its recognition site in the cytochrome oxidase subunit Vb [MCOSb +13/+33] gene [Virbasius et al. 1993] were unaffected by the addition of anti-NRF-1 or preimmune serum to binding reactions [lanes 10–12]. These results provide a direct link between the NRF-1 cDNA product and the NRF-1-binding activity present in HeLa cells.

**DNA-binding specificity of recombinant NRF-1**

The recombinant protein should display the same DNA-
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binding properties ascribed previously to NRF-1 [Evans and Scarpulla 1989, 1990; Chau et al. 1992]. Recombinant NRF-1 was thus used for competition DNase I footprinting of the rat cytochrome c promoter region [Fig. 4]. In the absence of competitor, the recombinant protein yielded several intense enhanced cleavages at the 5' and 3' ends of the footprint, with the absence of cleavages throughout the intervening protected region [Fig. 4, lane 3]. This pattern is identical to that observed previously using preparations of HeLa NRF-1 [Evans and Scarpulla 1990]. The footprint was eliminated by the inclusion of an excess of unlabeled oligonucleotides of previously characterized NRF-1 sites from nuclear genes with products that function in the mitochondria. These include cytochrome c (RC4, lane 4), cytochrome oxidase subunit VIc (COXVic, lane 5), and mouse MRP RNA [mMRP, lane 6]. Moreover, a sequence from the cytochrome c gene with two mismatches from the NRF-1 consensus did not compete (hCC1, lane 9), but a mutated derivative, active in both NRF-1 binding and transcriptional activity [Evans and Scarpulla 1990], did [hCC_UP, lane 10].

In addition to the known NRF-1 sites, we observed strong similarities to the NRF-1 consensus in recently isolated genes encoding cytochrome oxidase subunit Vb (COXVb) [Basu and Avadhani 1991] and mtTFA [Tomina et al. 1992]. Oligomers of each of these sites were tested for their ability to stimulate the activity of a truncated cytochrome c promoter in transfected cells. The COXVb -109/-87 and mtTFA -73/-46 oligomers stimulated promoter activity 12.3±3.4-fold and 6.0±1.9-fold,

Figure 1. Nucleotide and predicted amino acid sequence of the HeLa NRF-1 cDNA 5'-untranslated and coding region. Amino acid sequences matching those of tryptic peptides from the purified NRF-1 protein are underlined. The sequence co-incident with the PCR product amplified from HeLa cDNA using primers derived from the NRF-1 peptide sequences is indicated with a bold underline. The complete 2970-nucleotide NRF-1 cDNA sequence has been submitted to GenBank under accession number L22454.

Figure 2. Expression and purification of recombinant NRF-1. Coomassie-blue stained SDS-PAGE of molecular mass standards [lane 1] and 50 μl of log-phase culture of E. coli strain BL21(DE3) transformed with the NRF-1-coding region in the pET3d expression vector uninduced [lane 2] or induced by the addition of 0.4 mM IPTG [lane 3]. A lysate of an induced culture served as a negative control.
Figure 3. Recognition of HeLa NRF-1 by antiserum directed against the recombinant protein. Binding reactions contained 14 μg of HeLa nuclear extract (lanes 1–3, 10–12), 12 ng of affinity-purified HeLa NRF-1 (lanes 4–6), or 20 ng of bacterial NRF-1 heparin-agarose fraction (lanes 7–9). Labeled oligonucleotides contained either an NRF-1-binding site from the rat cytochrome c gene (RC4 -172/-147, lanes 1–9) or an NRF-2-binding site from the mouse COXVb gene (Virbasius et al. 1993) (lanes 10–12). Following the binding reaction, 1 μl of preimmune serum or goat antiserum was added to bacterially produced NRF-1, and the complexes were subsequently resolved on a native polyacrylamide gel.

Figure 4. Binding of recombinant NRF-1 to the rat cytochrome c promoter region. An end-labeled RC4 promoter fragment containing the NRF-1-binding site was subjected to DNase I digestion following incubation in a mixture without added protein (lane 2) or with the addition of 20 ng of NRF-1 heparin-agarose fraction (lanes 2–11). Competitor oligonucleotides indicated above lanes 4–11 were added at a 200-fold molar excess before the addition of the labeled fragment. The extent of the NRF-1 footprint is indicated by the vertical bar at right. [G] G reaction of the labeled fragment.
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The cytochrome c promoter has multiple cis-acting elements and therefore does not show complete dependence on NRF-1 for its activity (Evans and Scarpulla 1989). To enhance the NRF-1-dependent signal, four tandem sites from the cytochrome c (4XRC4) or the MRP promoter (Evans and Scarpulla 1989). The results demonstrate that a functional NRF-1 site is required for activation of transcription by the recombinant protein (Fig. 6A, lanes 2–4). Significant stimulation was observed using 0.1 µg of NRF-1, whereas 0.4 µg was inhibitory. This inhibition likely results from the competitive displacement of transcription complexes from the promoter template at high NRF-1 concentrations. In contrast, the linker insertion mutation resulted in a reduced level of transcription and completely eliminated activated expression (lanes 5–7). The transcripts were initiated at the same position observed for the in vivo cytochrome c transcripts in liver RNA (lane 1), indicating that they accurately reflect promoter activation through the normal initiation complex.

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Figure 5. Recognition of NRF-1-binding sites by recombinant NRF-1 through characteristic guanine contacts. Fragments containing representative NRF-1-binding sites from the indicated promoters were labeled on upper or lower strands, partially methylated, and subjected to preparative scale mobility retardation using recombinant NRF-1. Free DNA (F) and DNA isolated from bound complexes (B) were cleaved with piperidine, and the products were analyzed on denaturing gels. (●) Guanosine bases that (when methylated) strongly inhibit NRF-1 binding; (○) partial interference. Summarized below are the DNA sequences of each site and the positions of guanine nucleotide contacts compared with the consensus sequence and contacts derived from analysis of binding of HeLa NRF-1 to 10 known binding sites (Evans and Scarpulla 1990; Chau et al. 1992).

Figure 6. Transcriptional activation by recombinant NRF-1 through specific NRF-1 recognition sites. (A) In vitro transcription reactions with HeLa nuclear extract were carried out with 500 ng of a plasmid containing the RC4 promoter (lanes 2–4) or a promoter with a linker insertion disrupting the NRF-1-binding site (lanes 5–7). Heparin-agarose-purified bacterial NRF-1 was added as indicated (lanes 3,4,6,7). Transcription products were analyzed by primer extension and compared with the primer extension product of 20 µg of rat liver RNA (lane 1). (B) Products of in vitro transcription reactions using a truncated RC4 promoter construction RC4CAT/-66 (lanes 2–4) or the same promoter with four tandem copies of the RC4 (lanes 5–7) or mMRP (lanes 8–10) NRF-1-binding sites cloned upstream.
RNA [4XmMRP] promoters were cloned into an RC4 vector deleted of sequences upstream from −66, and the resulting constructs were used for in vitro transcription. Compared with the vector with no insert (Fig. 6B, lanes 2–4), those with the RC4 [lanes 5–7] or mMRP [lanes 8–10] NRF-1 sites displayed a strong, dose-dependent increase in transcription in response to added recombinant NRF-1. In this case, no inhibition was observed at 0.4 μg of NRF-1 because of the increased binding capacity of the promoter template for NRF-1. As with the intact cytochrome c promoter, initiation occurred at the same site used by the liver initiation complex in the synthesis of cytochrome c mRNA. These results establish that NRF-1 is a transcriptional activator that can function both in the proper promoter context and in a minimal promoter to direct the synthesis of high levels of accurately initiated transcripts.

NRF-1 has a new DNA-binding domain conserved in developmental regulatory factors

It was of interest to determine whether NRF-1 shares structural features with other proteins. A computer search revealed a region of extensive sequence similarity with two recently described developmental regulatory factors (Fig. 7). The first, P3A2, has been implicated in the correct expression of a cytoskeletal actin gene during sea urchin development (Calzone et al. 1991; Hoog et al. 1991). The second, the EWG gene product of Drosophila melanogaster participates in both nervous system and flight muscle development (Desimone and White 1993). Binding to DNA has been demonstrated for P3A2 but not EWG, and neither has yet been shown to function directly in transcriptional activation.

Alignment of NRF-1 with P3A2 and EWG reveals a stretch of striking sequence conservation among all three proteins between NRF-1 residues 65 and 284 (Fig. 7). This region coincides with the novel DNA-binding domain identified previously for P3A2 (P3A2 residues 25–258) [Hoog et al. 1991] and corresponding here to NRF-1 residues 61–290. In contrast, the three proteins share little similarity in their carboxy-terminal halves or in an amino-terminal extension present in NRF-1 and EWG.

To determine whether the highly conserved region coincident with the NRF-1 DNA-binding domain, a deletion series of truncated NRF-1 molecules [summarized diagrammatically in Fig. 8A] was expressed by in vitro transcription and translation, and the products were assayed for binding to radiolabeled RC4 −172/−147. As shown in Figure 8B, lane A, the intact cDNA yielded a translation product migrating at 68 kD. This protein was unaltered by deletion of the 3'-untranslated region to a position just downstream from the predicted NRF-1 translational terminator (lane B), confirming that the translation product is derived from the NRF-1 open reading frame. To demonstrate that the 68-kD translation product had the correct binding specificity, it was tested...
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for binding to known NRF-1-binding sites from nine different genes (Fig. 9). For each site, the major DNA–protein complex comigrated with the complex formed with affinity-purified NRF-1 (lanes 3–5). Minor discrepancies result from differences in lengths of the labeled oligomers. All NRF-1 complexes were competitively displaced by an excess of unlabeled RC4 –172/-147 but

Figure 8. Deletion mapping of the NRF-1 DNA-binding domain. (A) Schematic representation of in vitro-translated proteins tested for DNA binding. The shaded box represents the outer limits of the region required for DNA-binding activity. Construct A includes the complete cDNA sequence. In construct B all but 35 nucleotides of the 3'-untranslated region is deleted. C–H represent carboxy-terminal deletions of the residues indicated and were generated by either cleavage at a restriction endonuclease site in the native sequence [C,D,E,H] or insertion of a synthetic translation terminator [F,G]. In I the entire 5'-untranslated region, which includes several potential upstream initiators, was deleted, and J,K and L represent amino-terminal deletions of the indicated residues and restoration of the initiator ATG by the addition of an NcoI linker. Activity of the proteins in DNA-binding assays is summarized by + or − (right). (B) SDS-PAGE showing [35S]methionine-labeled translation products corresponding to the constructions diagrammed in A. Positions of molecular mass standards are at the left of each panel. (C) Electrophoretic mobility shift assay for binding activities of deletion mutants. Translation extracts containing the proteins deleted in A were incubated with end-labeled RC4 –172/-147 oligonucleotide, and the products were resolved on native acrylamide gels.

Figure 9. Specific binding of in vitro-transcribed and -translated NRF-1 to known NRF-1 recognition sites. Binding reactions contained 5 ng of affinity-purified HeLa NRF-1 (lanes 3–5) or 2 µl of wheat germ lysate without added RNA (lane 1), with RNA transcribed in vitro from an antisense NRF-1 template in pSG5 (lane 2), or with RNA transcribed from the NRF-1 sequence in the sense orientation (lanes 6–41). End-labeled oligonucleotides (10 fmole/lane) contained the NRF-1-binding sites designated above each panel. Lanes 1 and 2 also contained the labeled RC4 –172/-147 oligonucleotide. Binding reactions were carried out in the presence (+) or absence (−) of a 200-fold excess of unlabeled specific competitor (RC4 –172/-147) or a negative control oligonucleotide containing the rat cytochrome c ATF/CREB site [RC4 –281/-256]. DNA–protein complexes were resolved on 5% acrylamide native gels.
not by an excess of RC4 -281/-256 ATF/CREB oligomer. The faster migrating complex appears to result from a truncated product formed during in vitro synthesis, as indicated by the elimination of the lower complex by carboxy-terminal deletion to residue 305 (Fig. 8C, lane F).

Having confirmed the correct binding specificity, the carboxy-terminal deletions [Fig. 8A, constructs A-H] were expressed [Fig. 8B, lanes A-H] and assayed for DNA-binding activity (Fig. 8C, lanes A-H). DNA binding was unaffected until residues between 305 and 264 were removed [lanes F,G]. Likewise, when the aminoterminal deletions [Fig. 8A, constructs I-L] were expressed [Fig. 8B, lanes I-L] and assayed [Fig. 8C, lanes I-L], binding was lost on removal of residues 109-144 [lanes K,L]. The precise deletion of the 5'-untranslated region in construct I removes several potential initiation codons without affecting the translated product, further confirming the identity of the NRF-1 reading frame. The carboxy-terminal boundary of the DNA-binding domain, determined here between NRF-1 residues 264 and 305, compares favorably with that determined previously for P3A2 between NRF-1 residues 255 and 290. The aminoterminal boundary determined here between residues 109 and 144 is somewhat downstream from the P3A2 boundary between NRF-1 residues 61 and 126 but is overlapping in the region between residues 109 and 126. These results establish that the major region of sequence similarity among these proteins resides in their DNA-binding domains. Thus, NRF-1, P3A2, and EWG define a new family of regulatory factors that share a highly conserved DNA-binding motif.

Discussion

Identification of the cDNA-encoded product as NRF-1

Purification and molecular cloning of NRF-1 were undertaken as a requisite for further investigating its structural characteristics and biological functions. Previously, we had purified NRF-1 >30,000-fold from HeLa nuclear extracts and demonstrated that a single 68-kD polypeptide accounted for specific binding to the known NRF-1 sites [Chau et al. 1992]. The tryptic peptide sequences described here were derived from ~50 pmoles of the protein purified from >200 liters of HeLa cells.

The evidence presented here is consistent with the isolated cDNA encoding NRF-1. Both peptides derived from the purified protein were encoded in the cDNA, the expressed product of which migrated identically to HeLa NRF-1 on denaturing gels. Interestingly, the masses of NRF-1, P3A2, and EWG were all overestimated on denaturing gels by 30-50%, possibly reflecting shared structural features. Recombinant NRF-1 also binds specifically to the known NRF-1 sites through characteristic guanine contacts encompassing a single helical turn. Although the binding site is palindromic, the protein appears to bind as a monomer. Heterodimeric DNA–protein complexes were not detected when intact NRF-1 was mixed with derivatives truncated at either carboxyl or amino termini, and the mass of HeLa NRF-1 was estimated at ~52 kD by glycerol gradient centrifugation in both the presence and absence of its binding site [C. Virbasius, unpubl.]. Finally, in vitro transcription experiments unequivocally establish that the recombinant protein has the transcriptional activity and specificity expected for NRF-1. Taken together, the results presented here allow us to conclude with reasonable certainty that the cloned cDNA encodes NRF-1. However, given the existence of families of related transcription factors, it still remains a formal possibility that the true biological activity results from a protein that has escaped our detection.

Conservation of the NRF-1 DNA-binding domain in P3A2 and EWG

The striking conservation of the NRF-1 DNA-binding domain in P3A2 and EWG suggests that these proteins constitute a new family of regulatory factors with diverse functions in eukaryotic development. The P3A2 DNA-binding domain coincides with that defined here for NRF-1 and with the region of highest sequence conservation among all three factors. Although NRF-1 and P3A2 are acidic proteins with predicted isoelectric points of 4.71 and 5.49, respectively, nearly all of the lysine and arginine residues [33/34 for NRF-1 and 34/37 for P3A2] are clustered into two sequence blocks within the most highly conserved regions of the DNA-binding domain. The sequence between NRF-1 residues 89 and 160 [Fig. 7] is 25% lysine plus arginine and has 85% sequence conservation [identical plus similar residues] with P3A2. Likewise, the NRF-1 sequence between residues 199 and 274 is 20% lysine plus arginine and has 91% sequence conservation with P3A2.

In keeping with this structural conservation, the P3A2 recognition sites strongly resemble those for NRF-1. Both proteins make major groove contacts through alternating GC base pairs, and high-affinity binding occurs through a tandem repeat of the T/GCGGCA motif [Evans and Scarpulla 1990; Calzone et al. 1991]. An apparent difference is that P3A2 can bind a monomer of this sequence at reduced affinity, whereas stable binding of NRF-1 requires a tandem direct repeat of this half-site (Table 1). No NRF-1 binding was detected to sequences from the cytochrome c-like [hCC1 -454/-431] and COX-VIc [COXVIc -46/-20] genes containing perfect NRF-1 half-sites [Evans and Scarpulla 1990]. Two nucleotide changes in the hCC1 site that restore the direct repeat [hCC1UP -454/-431] also restore binding by NRF-1. This is confirmed here for these hCC1 sites using the recombinant protein [Figs. 4 and 9], making it unlikely that NRF-1 would bind with high affinity to several of the P3A2 target sites. Also, in the highest affinity P3A2-binding sites, the half-site motifs are separated by intervening nucleotides and, in one case, are rotated by one-half helical turn [Calzone et al. 1991]. These features have not been observed in the known NRF-1 recognition sites. It should be noted that P3A2 has been proposed to be a negative regulator of transcription through its dis-
Table 1.  

| Gene namea | Sequence | Locationb |
|------------|----------|-----------|
| I. Functional binding sites | | |
| rat cytochrome c | CCCGCATGGCGG | -166/-155 |
| human cytochrome c | CACCAGTGGCGG | -169/-158 |
| rat cytochrome c oxidase subunit VIc | CACGCATGGCGG | +39/+28 |
| mouse cytochrome c oxidase subunit Vb | GCAGCATTGGCG | -92/-103 |
| bovine cytochrome c oxidase subunit VIIa | TGGCGAAGGCGG | -11/-22 |
| human ubiquinone binding protein | TGGCGACGGCGG | -53/-64 |
| bovine ATP synthase \(\gamma\)-subunit | CGCGCAGGCGG | +4/+15 |
| mouse MRP RNA | CGCGCAGGCGG | -308/-297 |
| human MRP RNA | CGCGCAGGCGG | -293/-282 |
| human mitochondrial transcription factor A | GCGCGACGGCGG | -59/-70 |
| rat 5-aminolevulinate synthase | CGCGCAGGCGG | -77/-88 |
| rat tyrosine aminotransferase | CGCGCAGGCGG | -59/-48 |
| human eukaryotic initiation factor 2 \(\alpha\)-subunit | TCCGCATGGCGG | -37/-26 |
| Consensus | P gCGGAPgCGGCP | |
| II. Potential binding sites identified in GenBank/EMBL data basec | | |
| A. Metabolic enzymes | | |
| human arylsulfatase A | CGAGCAGGCGG | -71/-61 |
| human branched chain \(\alpha\)-keto acid dehydrogenase | TCCGCATTGGCG | -149/-138 |
| human carbonyl reductase | CGCGCAGGCGG | -46/-57 |
| human protein disulfide isomerase/prolyl 4-hydroxylase \(\beta\) | CGCGCAGGCGG | -79/-90 |
| human \(\alpha\)-enolase | CGCGCAGGCGG | -734/-723 |
| rat glutamate dehydrogenase | CGAGCATTGGCG | -227/-216 |
| human steroid 5\(\alpha\)-reductase | TGGCGAAGGCGG | -269/-280 |
| mouse ornithine decarboxylase | TGGCGAAGGCGG | -266/-277 * |
| human ornithine decarboxylase | CGCGCAGGCGG | -333/-344 * |
| rat ornithine decarboxylase | CGCGCAGGCGG | -268/-279 * |
| rat fatty acid synthase | CGCGCAGGCGG | -610/-621 |
| rat Na\(^+\)/K\(^+\) ATPase \(\alpha\)-1 subunit | CGCGCAGGCGG | -562/-549 * |
| human calcium-activated neutral protease | TGGCGATGGCGG | -18/-7 |
| human cathepsin D | GGCAGCAGGCGG | -175/-186 |
| | | |
| B. Signal transduction | | |
| mouse GM-CSF | CGCGCAGGCGG | -981/-992 |
| mouse hepatocyte growth factor-like protein | CGCGCAGGCGG | -1014/-1003 * |
| rat dopamine D1 receptor | CGACGAGGCGG | -1066/-1077 |
| human insulin receptor | GGCAGCAGGCGG | -1036/-1025 |
| human insulin-like growth factor receptor | GGCAGCAGGCGG | -29/-40 |
| human interferon receptor | GGCAGCAGGCGG | -85/-96 |
| human cyclophillin | TGGCAGATTGGCG | -173/-162 * |
| human lipoprotein receptor-like protein | GGCAGCAGGCGG | -151/-140 |
| rat calmodulin II \(\alpha\) | GGCAGCAGGCGG | -209/-220 * |
| human calretinina | GGCAGCAGGCGG | -197/-208 * |
| human Go-\(\alpha\) | GGCAGCAGGCGG | -53/-42 |
| human ADP ribosylation factor | GGCAGCAGGCGG | -835/-846 |
| human protein phosphatase 2A \(\alpha\) | GGCAGCAGGCGG | -215/-204 |
| mouse cyclic nucleotide phosphodiesterase | TGGCAGATTGGCG | -79/-90 |
| C. Chromosome maintenance and nucleic acid metabolism | | |
| human DNA polymerase \(\alpha\) | TGGCAGAAGGCGG | -235/-246 |
| human topoisomerase I | GGCAGCAGGCGG | -19/-8 |
| human H1 RNA \(\beta\) | GGCAGCAGGCGG | -145/-156 |
| human hnRNP core protein A | TGGCAGAAGGCGG | -558/-569 * |
| mouse S16 ribosomal protein | TGGCAGAAGGCGG | -521/-510 |
| mouse histone H2A \(\alpha\) | TGGCAGAAGGCGG | -398/-401 * |
| mouse histone H3 | GGCAGCAGGCGG | -35/-46 * |
| mouse histone H3 | GGCAGCAGGCGG | -40/-51 |

(Table 1 continued on following page.)
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Table 1. NRF-1-binding sites (Continued)

| Gene name   | Sequence                             | Location |
|-------------|--------------------------------------|----------|
| human RCC1  | TGGCGACGCGCA                          | -78/ -67* |
| human cdc2  | CCAAGTCGGCGCA                         | -717/ -706|
| D. Other    |                                      |          |
| human bcl-2 | CACGCGACGCGCA                         | -889/ -900|
| human GADD153 | GGCGACGCGCA                           | -175/ -186*|
| hamster GADD153 | GGCGACGCGCA                          | -865/ -354*|
| human 86-kD heat shock protein | GGCGACGCGCA | -286/ -257 |
| human synapsin 146 | TCGGCAGGCGCC | -241/ -252 |
| rat synapsin 46 | TGGGCAGGCGCC | -240/ -251 |
| mouse myb proto-oncogene | GGCGACGCGCA | -295/ -306 |

*The search was confined to primate and rodent genes. For part I, references are given for the demonstration of NRF-1 binding. For part II, publications of the gene sequence are cited.

Coordinates are given relative to the first transcription start site, if known. Otherwise the authors’ numbering system is followed. In some cases, the reverse complement of the published sequence is given to conform to the consensus. Sites identical to those of known function are indicated by an asterisk (*).

Rodent and primate sequences in the GenBank [release 76] and EMBL data bases [release 34] were searched with the Findpatterns program (Genetics Computer Group Manual, 1991), allowing one mismatch to the consensus given. Only mismatches found in the known sites in part I were allowed in a further screen of the identified sequences. Furthermore, only sites in upstream regions of published genomic sequences were included in the table.

References: 1(Evans and Scarpulla 1989), 2(Evans and Scarpulla 1990), 3(this work), 4(unpubl.), 5(Chau et al. 1992), 6(Braidotti et al. 1993), 7(Kreyssing et al. 1990), 8(Mitsubuchi et al. 1991), 9(Forrest et al. 1991), 10(Tasanen et al. 1992), 11(Giallongo et al. 1990), 12(Das et al. 1993), 13(Jenkins et al. 1991), 14(Katz and Rahana 1988), 15(Mosher et al. 1992), 16(Wen et al. 1989), 17(Amy et al. 1990), 18(Yagawa et al. 1990), 19(Miyake et al. 1986), 20(Cavaillles et al. 1993), 21(Miyatake et al. 1985), 22(Degen et al. 1991), 23(Zhou et al. 1992), 24(Tewari et al. 1989), 25(Mamula and Goldfine 1992), 26(Lutfalla et al. 1992), 27(Haendler and Hofer 1990), 28(Kutt et al. 1989), 29(Nojima 1989), 30(Parmentier and Lefort 1991), 31(Tsukamoto et al. 1991), 32(Lee et al. 1992), 33(Khew-Goodall et al. 1991), 34(Kurihara et al. 1990), 35(Miyake et al. 1990), 36(Miyatake et al. 1985), 37(Baer et al. 1990), 38(Biamonti et al. 1989), 39(Wagner and Perry 1985), 40(Hurt et al. 1989), 41(Sittman et al. 1983), 42(Furuno et al. 1991), 43(Ku et al. 1993), 44(Adachi and Tsujimoto 1990), 45(Park et al. 1992), 46(Luethy et al. 1990), 47(Walter et al. 1989), 48(Sauerwald et al. 1990), 49(Bender and Kuehl 1986).

placement of a zinc finger protein that binds the same sequence [Hoog et al. 1991]. In contrast, NRF-1 clearly functions as a positive activator of transcription. Thus, it remains to be determined whether the structural conservation between P3A2 and NRF-1 in their DNA-binding domains will be precisely reflected in their binding and transcriptional specificities.

The EWG protein is required for viability of Drosophila embryos and for the proper development of the embryonic nervous system [Desimone and White 1993]. Its molecular mass of 116 kD on denaturing gels is greater than that observed for P3A2 (62 kD) and NRF-1 (68 kD) and exceeds the mass predicted by its amino acid sequence (77 kD). Like P3A2, the sequence conservation with EWG is largely confined to the NRF-1 DNA-binding domain. Although its nuclear localization and structural conservation with P3A2 and NRF-1 are consistent with a function in gene regulation, there is yet no evidence for DNA-binding or transcriptional effects, nor have potential target genes been identified.

**NRF-1 and the nuclear control of mitochondrial function**

The mitochondrion serves to compartmentalize diverse cellular metabolic systems largely regulated by enzymes encoded in the nuclear DNA. The apparatus for electron transport and oxidative phosphorylation is unique in that both nuclear and mitochondrial genomes contribute protein subunits [Attardi and Schatz 1988; Clayton 1991; Wallace 1992]. The sole purpose of the mitochondrial genetic system is to complement the contribution of nuclear genes in maintaining respiratory function. Such interplay between the two genomes might involve novel pathways of intracellular communication.

One possibility is that NRF-1 may help to coordinate the expression of respiratory chain subunits with components of the mitochondrial transcription and replication machinery. Such a model is consistent with the finding of functional NRF-1 recognition sites in genes encoding respiratory subunits, the MRP RNA [Evans and Scarpulla 1990; Chau et al. 1992] and mtTFA. The latter two have the capability of communicating changes in nuclear gene expression to the mitochondria through their effects on mitochondrial DNA replication and transcription. In keeping with this hypothesis, we have recently established that the proximal promoter for the mtTFA gene is almost completely dependent on a NRF-1 recognition site for its activity both in transfected cells and in an in vitro transcription assay using recombinant NRF-1 [Virbasius and Scarpulla 1994]. NRF-1 control over mitochondrial function is also supported by the recent observation that the activity of 5-ALA synthase gene promoter is highly dependent on tandem NRF-1 recognition sites [Braidotti et al. 1993]. Thus, NRF-1 con-
control of this nuclear gene may serve to regulate the levels of the heme cofactor required by the respiratory cytochromes encoded by both genomes. These observations constitute a compelling case for an important integrative function for NRF-1 in communication between nuclear and mitochondrial genetic compartments.

Other regulatory functions for NRF-1 in coordinate gene expression

Our interest in nucleus-encoded mitochondrial functions, along with the remarkable consistency in the appearance of NRF-1 sites in the majority of genes in this category, has led to a direct functional characterization of these sites [Table 1]. However, the identification of NRF-1 sites in the tyrosine aminotransferase and eIF-2α genes suggested a broader integrative function for NRF-1 [Chau et al. 1992] and prompted a systematic search for potential binding sites in published gene sequences [Table 1]. The sites listed have only a single mismatch with the consensus derived from the 14 tested binding sites, and in each case, the mismatch is known to be allowed at that position in the functional sites. These imposed constraints in selecting potential binding sites make it likely that the genes containing these sites are targets for NRF-1. In fact, 14 of the 48 putative target genes in Table 1 have NRF-1 sites identical to those of known function. It should be emphasized, however, that the effects of NRF-1 on basal promoter activity are influenced by promoter context. For example, mutation of the NRF-1 sites in the cytochrome c and COXVIc genes [Evans and Scarpulla 1989, 1990] results in a more modest effect on promoter activity than mutation of the NRF-1 sites in the mtTFA [Virbasius and Scarpulla 1994] or 5-ALA synthase genes [Braidotti et al. 1993]. Also, the conservation of the NRF-1 DNA-binding domain in P3A2 and EWG suggests that this domain may be conserved in a mammalian family of related factors that mediate different biological functions through similar recognition sites. Thus, a rigorous analysis ultimately requires an evaluation of the NRF-1 sites within the proper promoter context and the identification of the cognate activator protein.

With these caveats in mind, some interesting observations emerge from Table 1. In addition to the cytochrome c and MRp RNA genes, there are several genes [ornithine decarboxylase, GADD153, and synapsin I] where the NRF-1 site is conserved in a similar location in different species. A majority of the genes are ubiquitously expressed, consistent with the wide distribution of the NRF-1-binding activity. In cases where ubiquitous and tissue-specific members of a gene family exist [cytochrome c, 5-ALA synthase, enolase, and the Na/K ATPase], NRF-1 sites are detected only in the widely expressed gene, suggesting involvement of NRF-1 in general, rather than tissue-specific cellular functions. Among a variety of metabolic enzymes encoded by these genes, several [ornithine decarboxylase and the branched-chain a-keto acid dehydrogenase] catalyze the rate-limiting step of their pathways [polyamine synthesis and branched-chain amino acid catabolism, respectively]. This is also true of tyrosine aminotransferase, 5-ALA synthase, and eIF-2α, supporting a role for NRF-1 in integrating a variety of metabolic pathways by modulating the expression of a key activity. Putative NRF-1 sites in genes encoding a number of receptors and components of signal transduction networks may indicate a role in the establishment or maintenance of regulatory systems that influence these or other cellular functions. Also prominent in the list are genes involved in chromosome maintenance and nucleic acid metabolism. This may reflect a requirement for coordinating the expression of the replication, transcription, and translation machinery with organelle biogenesis under certain conditions. Similarly, NRF-1 sites are found in genes that may be directly involved in cell cycle regulation (cdc2, RCC1) or are regulated by cell growth [ornithine decarboxylase, DNA polymerase-α, and GADD153]. Maintenance of mitochondria might be expected to require sensitivity to proliferative signals, and it is tempting to speculate that NRF-1 may function in transducing such signals. Thus, although the best defined biological role for NRF-1 is in the nuclear control of mitochondrial function, the NRF-1 protein or related proteins having the NRF-1 DNA-binding domain may have the potential for integrating diverse functions required for cell maintenance, growth, and proliferation.

Materials and methods

Purification and amino acid sequencing of NRF-1

DEAE-agarose and heparin-agarose fractionation of HeLa nuclear extracts have been described [Virbasius et al. 1993], except NRF-1 fractions were eluted with HEPES-D, 0.45 M KCl, diluted to 0.1 M KCl with HEPES-D, 0.1% NP-40, and loaded onto a NRF-1-specific affinity column as described [Chau et al. 1992]. Affinity-purified NRF-1 was isolated by SDS-PAGE, transferred to nitrocellulose, and the NRF-1 band was identified by Pon- ceau-S staining [Aebbersold et al. 1987]. In situ trypic digestion and peptide sequencing was performed by William S. Lane (Harvard Microchemistry Facility, Cambridge MA).

Amplification of NRF-1 sequence and cDNA library screening

One sense primer and two sets of antisense primers were used to amplify the NRF-1-coding sequence. One set of primers, 5'-GCIGAICATTGCCCGCCCGCGCAIAGTIAACTCT-3' derived from the NRF-1(72) peptide and 5'-GCCTGNGTCCANAGAAC-3' derived from the NRF-1(38) peptide, yielded a PCR product. Briefly, cDNA was synthesized using AMV reverse transcriptase [Promega] with 2 μg of oligo(dT)-primed HeLa poly(A) RNA in a total volume of 20 μl. The product (2 μl) was mixed with two different pairwise combinations of sense and antisense primers and amplified with AmpliTag DNA polymerase [Perkin-Elmer Cetus] for 50 cycles [94°C for 1 min, 50°C for 2 min, 72°C for 2 min]. The products were ligated to M13mp18 for sequencing. A 269-bp PCR product, encoding portions of the two NRF-1 peptides, was subcloned into pGEM4 Blue. The insert was labeled by nick translation for screening a HeLa cDNA library in λZIP11 [a gift of Dr. R. Morimoto, Northwestern University, Evanston, IL]. The 3-kb insert of one of two positive

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Plasmid constructions

The full-length NRF-1 cDNA was put into the pSG5 expression vector (Green et al. 1988) in the sense orientation by ligating the upstream EcoRI–PstI fragment and downstream PstI–BamHI fragment of pGEM7zf(NRF1) into the EcoRI and BamHI sites of pSG5 to produce pSGSNRF1/1–1662. The antisense construction was generated by ligating the Xhol–BamHI fragment into the same sites of a pSG5 vector modified by insertion of a Xhol linker at the EcoRI site. pSGSNRF1/1–1662 [construct B in Fig. 8A] was generated by addition of a BamHI linker at the AccI site downstream of the termination codon, removing the 3′ untranslated region, pSGSNRF1/1–1662 and pSGSNRF1/1–908 [constructs F and G in Fig. 8A] were generated by exonuclease III digestion from the AccI site, followed by addition of an Asp718I linker and cloning into a pGEM7zf (+) containing a synthetic universal translation terminator (Pharmacia) in its SmaI site. Deleted fragments were recloned to pSG5 using flanking PstI and BamHI sites. Amino-terminal truncations were generated either by PCR cloning or restriction enzyme cleavages. Briefly, pSGSNRF1/1–1662 [construct J in Fig. 8A] was generated by PCR using a sense primer, CCATGGGAATTCGGCGCCG, and an antisense primer, CCACCGCAATAATCCATGGTCGAC and used to transform E. coli BL21(DE3). Partial purification of the overexpressed protein was described (Pogonone et al. 1991). The ammnonium sulfate fraction was diluted 10-fold with HEPES-D and applied to a 1-mL heparin–agarose column in HEPES–D, 0.1 M KC1. NRF-1 was eluted in a 0.1–1 M KC1 gradient. Goat anti-NRF-1 serum was used to raise the heparin–agarose peak fraction (East Acres Biologicals, Southbridge, MA).

Methylation interference and footprinting

Methylation interference and DNase I footprinting were described previously (Evans and Scarpulla 1990). A 130-ng recombinant NRF-1 ammonium sulfate pellet was used in the preparative shift of methylated fragments, and a 20-ng NRF-1–heparin–agarose fraction dialyzed to 0.1 M KC1 was used in footprinting. When indicated, a 200-fold excess of NRF-1–specific or –nonspecific competitors was added before the addition of labeled fragment in footprinting.

Expression and purification of the recombinant NRF-1 and antisera preparation

NRF-1 was expressed using the T7 expression system (Studet et al. 1990). An Ncol site was introduced at the NRF-1 initiation codon by PCR using a sense primer, GAATTCATGGAACAC, and the same antisense primer as above. The PCR product was digested with Ncol and PstI to give a 221-bp fragment, ligated with a PstI–BamHI fragment containing the rest of NRF-1-coding region to the Ncol and BamHI sites of pET3d and used to transform E. coli BL21(DE3). Partial purification of the overexpressed protein was described (Pogonone et al. 1991). The ammonium sulfate fraction was diluted 10-fold with HEPES-D and applied to a 1-mL heparin–agarose column in HEPES–D, 0.1 M KC1. NRF-1 was eluted in a 0.1–1 M KC1 gradient. Goat anti-NRF-1 serum was used to raise the heparin–agarose peak fraction (East Acres Biologicals, Southbridge, MA).

In vitro transcription, translation, and mobility shift assay

The pSG5 vectors described above include a T7 promoter upstream of the cloning site. To generate runoff transcription templates, pSGSNRF1/1–2970 was linearized with BamHI to generate the full-length [construct A in Fig. 8A], Ncol [C, 5′-476–503], EcoRI [D, 449–503], Bgl II [E, 531–503], or EcoRV [H, 238–503]. The other carboxy-terminal and all amino-terminal deletions were linearized with BamHI. In vitro transcription was performed by using T7 polymerase (Promega) and RNA translated in wheat-germ extract (Promega). Reactions contained unlabeled methionine for use in mobility-shift assays or [35S]methionine for analysis of the protein products on SDS–polyacrylamide gels. Mobility shift assays were done as described (Evans and Scarpulla 1990). Binding reactions contained 1 μg of sonicated calf thymus DNA and 2 μg of BSA in HEPES-D, 100 mM KC1. When indicated, a 200-fold excess of NRF-1–specific or –nonspecific competitors was added. In addition to oligonucleotides described previously (Evans and Scarpulla 1990, Chau et al. 1992), the following oligonucleotides were employed in binding assays:

In vitro transcription

In vitro transcription and analysis of transcripts were done as described (Virbasius et al. 1993), except 54 μg of HeLa nuclear extract and 0.5 μg DNA template were used. The recombinant NRF-1 used was the dialyzed heparin–agarose peak fraction. Constructions used as templates have been described (Evans and Scarpulla 1989, 1990).

Transient transfection

For transfection, 3 × 10⁵–4 × 10⁶ COS cells were resuspended in 0.8 ml of ZAP buffer (20 mM HEPES, 137 mM NaCl, 0.5 mM KC1, 0.7 mM Na2HPO4, 6 mM dextrose adjusted to pH 7.05) and mixed with 5 μg of reporter plasmid and 15 μg of pGEM4blue carrier. The cells were then subjected to a single pulse (270 V, 960 μF) using a Bio-Rad Gene Pulser. The cells were harvested after 48 hr, and extracts were analyzed for chloramphenicol acetyltransferase (CAT) activity and CAT-coding DNA in Hirt supernatants as described previously (Evans and Scarpulla 1988). The reporter plasmids used were either RC4CATBA/-66BA or RC4CATBA/-66BA with NRF-1 oligonucleotides from MCoSb, mtTFA, or RC4 cloned upstream as described

For antisemur supershift experiments, 1 μl of anti-NRF-1 or preimmune serum was added to binding reactions that had been incubated for 15 min as described above. After an additional 15 min of incubation, the reaction was fractionated by electrophoresis on 4% (58:1) acrylamide/bisacrylamide gels.
previously [Evans and Scarpulla 1990]. Values were the average of six separate determinations ± s.d.

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