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Nuclear Respiratory Factors 1 and 2 Utilize Similar Glutamine-Containing Clusters of Hydrophobic Residues To Activate Transcription

SAJIV GUGNEJA, CHING-MAN A. VIRBASIUS,† AND RICHARD C. SCARPULLA*

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

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Nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) are ubiquitous transcription factors that have been implicated in the control of nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication. Recently, both factors have been found to be major transcriptional determinants for a subset of these genes that define a class of simple promoters involved in respiratory chain expression. Here, functional domains required for transactivation by NRF-1 have been defined. An atypical nuclear localization signal resides in a conserved amino-terminal region adjacent to the DNA binding domain and consists of functionally redundant clusters of basic residues. A second domain in the carboxy-terminal half of the molecule is necessary for transcriptional activation. The activation domains of both NRF-1 and NRF-2 were extensively characterized by both deletion and alanine substitution mutagenesis. The results show that these domains do not fall into known classes defined by a preponderance of amino acid residues, including glutamines, prolines, or isoleucines, as found in other eukaryotic activators. Rather, in both factors, a series of tandemly arranged clusters of hydrophobic amino acids were required for activation. Although all of the functional clusters contain glutamines, the glutamines differ from the hydrophobic residues in that they are inconsequential for activation. Unlike the NRF-2 domain, which contains its essential hydrophobic motifs within 40 residues, the NRF-1 domain spans about 40% of the molecule and appears to have a bipartite structure. The findings indicate that NRF-1 and NRF-2 utilize similar hydrophobic structural motifs for activating transcription.

The oxidative activity of the mitochondrion relies upon the functional interplay of gene products from two physically distinct genetic systems, nuclear and mitochondrial (2). The vertebrate mitochondrial genome is extremely compact, and its protein coding capacity is limited to only 13 subunits of the inner membrane respiratory complexes. Thus, although these subunits are essential to oxidative phosphorylation, the mitochondrial oxidative pathways mainly comprise the products of nuclear genes. Moreover, aside from the mitochondrially encoded rRNAs and tRNAs, nuclear genes contribute all other components of the mitochondrial genetic system and thereby govern the expression and replication of the mitochondrial genome (7).

In recent years, the analysis of nuclear respiratory genes from vertebrate sources has resulted in the identification of a number of candidate transcription factors that may contribute to the nuclear control of mitochondrial function (27). In particular, recognition sites for nuclear respiratory factors (NRFs) 1 and 2 have been found in many mammalian nuclear genes whose products function in the mitochondria (31). These genes encode subunits of the respiratory chain complexes, the rate-limiting enzyme of heme biosynthesis, and mitochondrial DNA transcription and replication factors (4, 35, 38). These findings led to the hypothesis that NRF-1 and NRF-2 may help govern nuclear-mitochondrial interactions by coordinating the expression and/or function of respiratory subunits from both genomes (31, 37).

Although the subsets of respiratory target genes initially defined by NRF-1 and NRF-2 were nonoverlapping, several such genes that utilize both factors to optimize their expression have now been identified (32, 37, 38). The activities of two cytochrome oxidase subunit promoters, COXVb (38) and COXVIIaL (32), have been found to require the specific binding of both factors. Likewise, the nuclear gene encoding mitochondrial transcription factor A, a protein that participates in the transcription and replication of mitochondrial DNA (33), is highly dependent upon both NRF-1 and NRF-2 recognition sites for expression (37). In two of these promoters, mtTFA and COXVIIaL, the binding of NRF-1 appears to facilitate the contribution of other factors such as Sp1 and NRF-2 (32, 37). Mutation of the NRF-1 site in the mtTFA promoter markedly reduces the influence of Sp1 and NRF-2 on promoter function (37). In the COXVb promoter, synergism among multiple tandemly arranged NRF-2 sites is essential for promoter activity (38). The promoters of all three genes are very compact, and the NRF-1 and NRF-2 sites are clustered within approximately 100 nucleotides of the transcription start sites. Thus, the mtTFA, COXVb, and COXVIIaL genes are related both by their essential contribution to mitochondrial function and by having relatively simple promoters that are highly dependent on NRF-1 and NRF-2.

NRF-1 is one of three recently described regulatory proteins that are related by a novel DNA binding domain (12, 20, 35). In contrast to one family member, P3A2, which is a negative regulator of transcription (20), NRF-1 clearly functions as a transcriptional activator. A third family member, the erect wing gene product, is required for neuromuscular development in *Drosophila melanogaster* (12). The disparate functions of the

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, Northwestern University Medical School, 303 East Chicago Ave., Chicago, IL 60611. Phone: (312) 503-2946. Fax: (312) 503-0798.

† Present address: Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605.

three proteins are consistent with the lack of sequence similarity outside their DNA binding domains (35). Here, a systematic analysis of the domains required for transactivation by NRF-1 has been performed. The results show that in addition to the previously defined DNA binding domain (35), the molecule consists of two distinct modules required for nuclear localization and transcriptional activation. The transcriptional activation domains of both NRF-1 and NRF-2 were analyzed by extensive alanine substitution mutagenesis. These domains differ from other activators in that they are not rich in a predominant amino acid residue. However, they both require a combination of specific hydrophobic motifs for function.

MATERIALS AND METHODS

Plasmid constructions. Plasmids expressing NRF-1 and deleted derivatives for transactivation and nuclear localization experiments were constructed from the pSG5NRF-1(119-1662) parent vector as described previously (35). Site-directed mutations of the nuclear localization signal (NLS) were generated by PCR using the following primers: 390S, 5′-AACGCGTCCGATACGGCCTC-3′; 390A, 5′-CGACGCGTTCCTGTTGCCAC-3′; 430S, 5′-CCATCTATCCGGAAGAGGCA-3′; 430A, 5′-GCCCCCTCGAGATAGATGG-3′; 462S, 5′-CCCATGGTTCGAGCCACGTTAG-3′; and 462A, 5′-AACCATGGGAAGCAAACGTG-3′. Each set of primers forms a sense (S) and antisense (A) pair, with numerical coordinates of both primers and vectors referring to nucleotide positions from the 5′ end of the original NRF-1 cDNA (35).

NRF-1 amino acid conversions are as follows: 89-Lys-Lys-Arg-Lys-Arg-93 to 89-Thr-Arg-Pro-Ile-Arg-93 by primer pair 390, 103-Ile-Arg-Lys-Arg-Gln-107 to 103-Ile-Ser-Arg-Gly-Gln-107 by primer pair 430, and 112-Leu-Arg-Lys-Leu-Arg-116 to 112-Leu-Pro-Met-Val-Arg-116 by primer pair 462. Primer 430S was used in conjunction with a downstream antisense primer, and 430A was used in conjunction with an upstream primer (5′-GAACTCCATGGAGGAACAC-3′) which incorporates an *Nco*I site at the initiator ATG in PCRs with pSG5NRF-1(119-1662) as the template. The products were cut with *Nco*I-*Xho*I and *Xho*I-*Hin*cII, respectively. The *Xho*I site is incorporated into the 430 primer pair, while the *Hin*cII site is natural. Both digested products were cloned together into the *Nco*I and *Hin*cII sites of pGEM5Z (Promega). A *Pst*I-*Hin*cII fragment from this clone was used to replace the same fragment in pSG5NRF-1(119-1662) to generate NRF-1 with the mutations indicated above. The same procedure was performed with primer pair 462 except that the PCR products were cut with *Pst*I-*Nco*I and *Nco*I-*Hin*cII and the digested PCR product pair was cloned into pGEM5Z cut with *Pst*I and *Hin*cII. The procedure was then repeated using primer pair 390 on wild-type NRF-1 or NRF-1 constructions previously mutated with primer pair 430 or 462. The PCR products were digested with *Nco*I-*Mlu*I or *Mlu*I-*Eco*RV, and the product pair was cloned into the *Nco*I and *Eco*RV sites of pGEM5Z. As before, the mutated *Pst*I-*Hin*cII fragment was used to replace the same region in pSG5NRF-1(119-1662) to generate the NRF-1 nuclear localization signal (NLS) mutants. All mutations were confirmed by DNA sequencing.

Gal4 fusion proteins were made in pSG424 (30), which expresses the Gal4 DNA binding domain, Gal4(1-147). Gal4/NRF-1 fusions were made by adding an *Xba*I linker at the 3′ *Bam*HI site of the appropriate pSG5NRF-1 construction previously described (35) to enable cloning into the *Xba*I site of the pSG424 polylinker. For fusion constructions starting at NRF-1 amino acid 145, a *Bam*HI linker was added at a natural *Dra*I site to enable cloning in the equivalent site in pSG424. Constructions with amino-terminal endpoints at NRF-1 amino acids 304, 338, 387, 431, and 466 were synthesized by PCR using the sense primers 5′-GAGGATCCAGACTGTAGTCC-3′, 5′-GGGATCCCAACCACGGTC-3′, 5′-TGGATCCCAGAGGCCGCAG-3′, 5′-CGGATCCTCTTGTCTGGG-3′, and 5′-GGGATCCGCCTCGCCCAG-3′ in which a *Bam*HI site has been incorporated such that the reading frame of NRF-1 is in frame with that of Gal4. The template DNA was the pSG5NRF-1 construction which ended at the desired 3′ end. The antisense primer used for most constructions was within pSG5 sequences past the desired endpoint which amplified an *Acc*65I site previously incorporated (35). Substitution of an Ala codon for Gln-308 was accomplished by PCR using the primer 5′-GAGGATCCAGACTGTAGTCGCGACTTTTAGT-3′, while substitution of Ala codons for those specifying hydrophobic amino acids in the cluster around Gln-308 was accomplished with the primer 5′-GAGGATCCAGACTGCAGCCCAGACTGCTAGTAACC-3′. Fusion constructions carboxy terminal to amino acid 337 ending at amino acid 503 were made with the antisense primer 5′-AGGTACCTGTTCCAATGTCACC-3′, which incorporates an *Acc*65I site for cloning. Fusion constructions terminating at amino acid 449 were made by using as the antisense primer 5′-CATCTAGACATTAGCATCTTGGAC-3′, which incorporates an *Xba*I site for cloning purposes.

The Gal4/NRF-2β_1(258-327) fusion used for further mapping of the NRF-2 activation domain was generated by PCR using a sense primer (5′-CGGGATCCTGGATGGTCCCA-3′) to incorporate a *Bam*HI site at the desired 5′ endpoint. To allow for *Bam*HI-*Xba*I cloning into pSG424, an antisense primer from beyond the pSG424 polylinker region in Gal4/β_1(10-327) (19), which contains an *Xba*I site, was used for amplification. Site-directed mutations in the cluster around NRF-1 Gln-308 are described above. To generate the Gal4 fusions containing other site-directed mutations within the NRF-1 and NRF-2β_1 activation domains, the *Bam*HI-*Acc*65I fragment from Gal4/NRF-1(338-477) and the *Bam*HI-*Xba*I fragment from Gal4/β_1(258-327) were cloned into M13, mutagenized by the method of Kunkel (23), and then recloned into pSG424. Junctions of all Gal4 fusion constructions and site-directed mutations were confirmed by DNA sequencing.

To generate the 4xNRF-1Luc reporter, plasmid pUHC 13-3 (17) was modified by partial *Eco*RI digestion to remove the regulatory region upstream of luciferase coding sequences. The promoter and intron fragments from 4xRC4(−172/−147)RC4CATBΔ/−66 (13, 35) were then cloned as two fragments (*Sac*I-*Hin*dIII and *Hin*dIII-*Bgl*II) into the *Sac*I and *Bam*HI sites within the multiple cloning region upstream of the luciferase coding sequences. To generate the G_5BLuc reporter, the regulatory region upstream of luciferase coding sequences in pUHC 13-3 (17) was removed by *Xho*I-plus-*Acc*65I digestion and replaced with the Gal4 binding site and E1b TATA box fragment from G_5BCAT (25) that was digested with the same enzymes.

Cell culture and transfection. Conditions for culture and transfection of COS-1 cells by the $CaPO_4$ precipitation method were essentially as described previously (16, 18, 36). For NRF-1 transactivation and Gal4 fusion experiments, cells on each 100-mm-diameter dish were transfected with 1 μg of pSG5NRF-1 or Gal4 fusion protein expression vector, 2 μg of 4xNRF-1Luc or G_5BLuc reporter, 100 ng of RSVLacZ (26), and 17 μg of sonicated calf thymus DNA as the carrier. Cells were harvested from triplicate plates 48 h posttransfection and pooled. Nuclear protein extracts were prepared from one-third of the cell pool as described elsewhere (1) to assay the expression of NRF-1 deletion derivatives or Gal4 fusion proteins with an NRF-1 binding or Gal4 binding oligonucleotide in a mobility shift assay (6, 19, 36). Cell extracts were made from the remaining two-thirds of the pool by freeze-thaw cycles. Extracts were assayed for luciferase activity in 350 μl of buffer containing the sample in 0.25 M Tris (pH 7.8), 15 mM $MgSO_4$, 5 μg of bovine serum albumin (BSA) per ml, and 2.5 mM ATP. Cell extracts were also assayed for β-galactosidase activity to normalize for transfection efficiency as described elsewhere (29).

Immunofluorescence microscopy. For NLS experiments, COS-1 cells were plated on coverslips and transfected with 2 μg of pSG5 NRF-1 expression vector with 18 μg of sonicated calf thymus DNA as described above. After 48 h, the coverslips were washed twice with phosphate-buffered saline (PBS) and cells were fixed in 3.7% formaldehyde in PBS for 5 min. After washing with PBS, cells were extracted with methanol at −20°C for 2 min and air dried. After wetting with PBS, the coverslips were incubated with rabbit anti-NRF-1 antiserum (1:1,000 dilution) in PBS containing 5% normal goat serum and 0.5% BSA for 1 h at 37°C. The coverslips were then washed in PBS and incubated with a fluorescein conjugated anti-rabbit immunoglobulin G mouse monoclonal antibody (Sigma) in PBS containing 0.5% BSA for 1 h at 37°C. Following incubation with the secondary antibody, cells were washed in PBS with a final wash in water and mounted onto microslides with a drop of 5% (wt/vol) *n*-propyl gallate in 90% glycerol–10% PBS. Slides were stored at −20°C in the dark until examined.

RESULTS

Resolution of the DNA binding domain from other functional domains by deletion mapping. NRF-1 was recently identified as a member of a family of transcription factors related by a novel DNA binding domain (35). The amino-terminal boundary of this domain was localized between residues 110 and 144, and the carboxy-terminal boundary was localized between residues 264 and 305. A high degree of sequence identity among NRF-1 and the other two members of the family, P3A2 (5) and EWG (12), extends beyond the amino-terminal boundary of the DNA binding domain to residue 64. This suggests that there are shared functional constraints operating on the region between residue 64 and the boundary of the DNA binding domain demarcated by the 110-to-144 interval. By contrast, no significant sequence similarity exists in the carboxy-terminal half of these proteins, suggesting that this portion of the molecule is functionally divergent among family members.

To systematically define functional regions of the NRF-1 protein flanking the DNA binding domain, cotransfections were performed with an NRF-1-expressing plasmid and a reporter plasmid that directs the NRF-1-dependent expression of luciferase through a promoter containing four tandem NRF-1 recognition sites (Fig. 1). Expression of full-length NRF-1 resulted in a sevenfold transactivation of the reporter in COS cells. A carboxy-terminal deletion to residue 477 gave

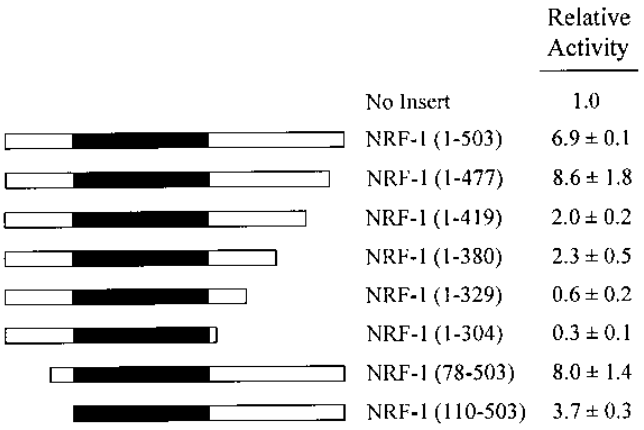

FIG. 1. Transactivation by NRF-1 deletion mutants. NRF-1 and various deletion mutants were tested for their ability to activate expression of a cotransfected luciferase reporter plasmid containing four NRF-1 binding sites. Filled boxes indicate the NRF-1 DNA binding domain. The activity of the reporter transfected with expression vector lacking an insert is defined as 1.0. Results are averages ± standard deviations of three determinations.

slightly increased activity compared with that of the full-length construct (Fig. 1). Further deletion to residue 419 resulted in approximately fourfold reduction in luciferase expression, suggesting that a functional determinant resided in the 419-to-477 interval. Deletion to residue 380 gave no change, but deletions to 329 and to 304 resulted in approximately three- to fourfold and twofold reductions in activity, respectively, suggesting the presence of a second functional domain between 380 and 304. The reduction in activity to levels below that of the no-insert control most likely results from competitive inhibition of the endogenous NRF-1. Deletion from the amino terminus to residue 77 yielded no reduction of activity, but further deletion to the boundary of the DNA binding domain at residue 109 resulted in a twofold loss. None of the deletion mutations had significant effects on the level of expression, as demonstrated by the recovery of very similar DNA binding activities in crude nuclear extracts from transfected cells. The maximal individual variation from the mean binding activity was less than 25% (data not shown). Thus, the regions identified contribute to functions other than DNA binding that are necessary for activation of the reporter gene.

Identification of signals for nuclear localization. The highly conserved region between residues 78 and 110 contains clusters of basic residues that are reminiscent of an NLS. Thus, the drop in activity associated with the deletion in this interval may result from altered subcellular localization of the protein. To examine this possibility, the subcellular distribution of the wild-type protein and several amino-terminal deletions was assayed by immunofluorescence after expression in COS cells. Expression from the transfected wild-type construct (Fig. 2, wild-type panel) was easily distinguished from the low background level of the endogenous NRF-1 (no-insert panel) and clearly resulted in a nuclear location. Likewise, expression of the protein with amino-terminal deletion to residue 77 (Del 1-77) was also exclusively nuclear. Further deletion to residue 109 gave a heterogeneous distribution of expression. The immunofluorescent staining was both nuclear and cytoplasmic in all of the transfected cells, but the cytoplasmic pattern of expression varied from being homogeneously distributed (not shown) to having a punctate staining pattern (Fig. 2, Del 1-109). This distribution is consistent with the fact that the construct was only half as active as the wild type and the 1-to-77 deletion in its ability to transactivate the reporter (Fig. 1). Further deletion to residue 144 (Fig. 2, Del 1-144) resulted in cytoplasmic localization, suggesting that signal redundancies reside between residues 110 and 144. Interestingly, this interval contains a basic cluster beginning at residue 110.

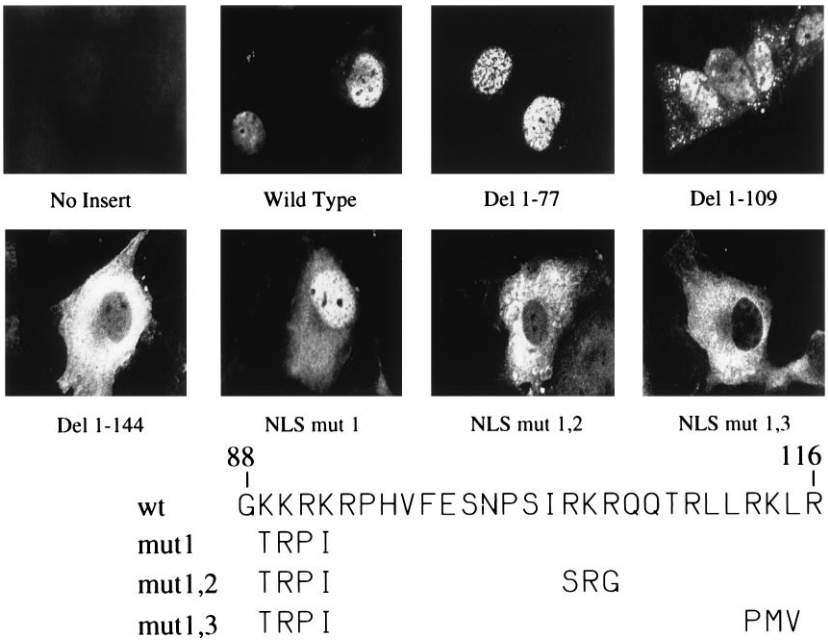

FIG. 2. Mapping of the NRF-1 NLS. COS-1 cells plated on coverslips were transfected with either empty vector (no insert), NRF-1 expression vector (wild type), or vectors expressing amino-terminal deletion (Del) or site-directed mutant variants (NLS mut) of NRF-1 as indicated. At 48 h posttransfection, cells were fixed and stained with rabbit anti-NRF-1 antiserum followed by fluorescein-conjugated anti-rabbit immunoglobulin G monoclonal antibody. Cellular distribution of NRF-1 and mutants was visualized by fluorescence microscopy. The amino acid sequences of the site-directed mutants are shown below. wt, wild type.

The sequence between residues 88 and 106 has two clusters of basic residues separated by 10 amino acids and therefore resembles a bipartite NLS (3). To test the contribution of the first and most basic of the clusters to the subcellular distribution of NRF-1, several nonbasic amino acids were introduced into the sequence Lys-Lys-Arg-Lys-Arg beginning at residue 89 (Fig. 2). The distribution of the resulting mutated molecule was mainly nuclear (Fig. 2, NLSmut1) with a significant portion detectable in the cytoplasm, suggesting that the first basic cluster contributes to efficient targeting to the nucleus. When NLSmut1 was combined with mutations in the second cluster of basic residues (NLSmut1,2), the expressed protein was again distributed in both the nucleus and cytoplasm. However, from one transfected cell to another, the ratio of nuclear to cytoplasmic staining varied from a homogeneous pattern (similar to NLSmut1) to a largely cytoplasmic distribution as shown. This result was similar to that obtained with the deletion from 1 to 109 but differed from the deletion from 1 to 144, which gave only cytoplasmic staining, again suggesting that a signal redundancy was present in the interval between 109 and 144. Mutations in a third basic cluster between 113 and 116 were therefore combined with NLSmut1 to give NLSmut1,3. This construct yielded cytoplasmic staining similar to that observed with the deletion from 1 to 144. Thus, this third cluster (residues 113 to 116) can account for the fact that deletion beyond residue 109 is required to completely eliminate nuclear staining. All of the NLSmut proteins were able to bind DNA (not shown), indicating that the differences in subcellular distribution were not the result of indirect effects of the mutations on DNA binding. These results are consistent with the conclusion that a major function of the conserved region upstream from the amino-terminal boundary of the DNA binding domain is to direct NRF-1 to its site of action within the nucleus.

Multiple functional motifs define a diffuse activation domain. The experiments reported in Fig. 1 demonstrate that distinct regions downstream from the DNA binding domain (304 to 380 and 419 to 477) have a marked effect on transactivation of the reporter. To further characterize these regions, it was desirable to increase the sensitivity of the assay by eliminating the background activation by endogenous NRF-1. This was accomplished by constructing a series of fusions to the *Saccharomyces cerevisiae* Gal4 DNA binding domain and using a luciferase reporter whose expression is driven by Gal4 binding sites. The fusions to Gal4 were constructed through NRF-1 amino acid 145, thus eliminating the NRF-1 NLS and DNA binding activity (Fig. 3). Carboxy-terminal deletions of the fusion proteins yield essentially the same results obtained with NRF-1 and its mutated derivatives, as shown in Fig. 1. The fusion proteins were expressed at equivalent levels, except for Gal4/NRF-1(145-329). This fusion was expressed at an approximately fivefold lower level than the others in this series, and therefore, much of the drop in activity most likely results from reduced expression. However, as shown in Fig. 1 [compare NRF-1(1-380) to NRF-1(1-329)], this region contributed to the ability of NRF-1 to transactivate, and this result was confirmed in subsequent Gal4 fusion constructs [Fig. 4, compare Gal4/NRF-1(338-503) to Gal4/NRF-1(387-503)].

To resolve further the functional determinants, fine deletions of the entire region were constructed as Gal4 fusions (Fig. 4). The Gal4 DNA binding activity recovered from each transfected cell extract varied less than 15% from the mean of all extracts (not shown) for most of the deletion mutants. The only exception was the 466-to-503 fusion, which was expressed at about 40% lower levels. However, this was insufficient to account for the substantially lower activity of this fusion protein. The highest activity was obtained with the fusion containing NRF-1 residues 304 to 477. The absence of the partial NRF-1 DNA binding domain in this construct resulted in fourfold more activity than that with the Gal4/NRF-1(145-503) fusion protein shown in Fig. 3, indicating that the presence of an incomplete DNA binding domain in 145 to 503 is apparently inhibitory. However, this inhibitory effect does not appear to be sequence specific, because removal of unrelated sequences from similar constructs containing the NRF-2β activation domain also stimulated activity in the context of a Gal4 fusion (19). Most of the activity observed in the 304-to-477 fusion was retained upon amino-terminal deletion to 338 (338 to 477). Subsequent carboxy-terminal deletion to residue 449 gave a nearly 30-fold reduction, thus refining the localization of a major functional determinant to between 449 and 477. Fur-

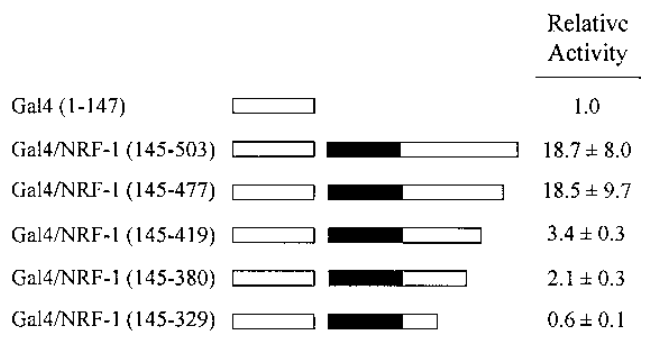

FIG. 3. Transactivation by Gal4/NRF-1 fusion proteins. The indicated regions of NRF-1 were fused to Gal4(1-147) and tested for transactivation of a cotransfected luciferase reporter containing five Gal4 binding sites. The Gal4 DNA binding domain (grey boxes), the remainder of the NRF-1 DNA binding domain (black boxes), and portions of the carboxy-terminal region of NRF-1 (open boxes) are indicated schematically. The activity of expressed Gal4(1-147) alone is defined as 1.0. Results are the averages ± standard deviations of three independent determinations.

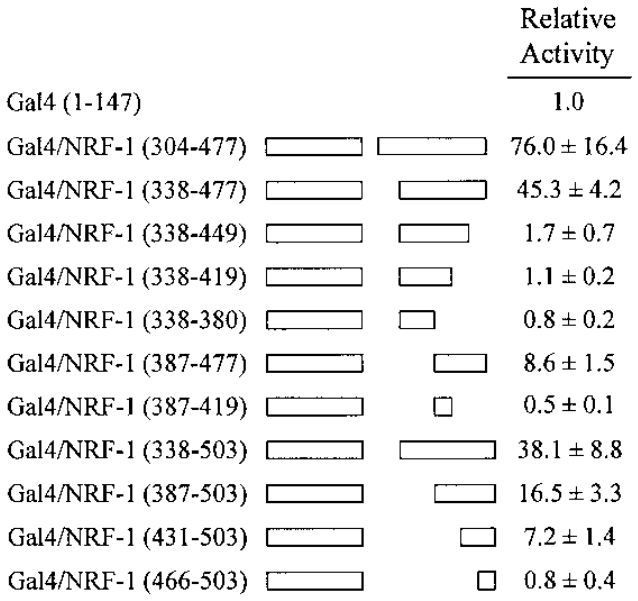

FIG. 4. Deletion fine mapping of the NRF-1 activation domain. Portions of the carboxy-terminal region of NRF-1 (open boxes) were fused to the Gal4 DNA binding domain (shaded boxes) and tested for their ability to transactivate a cotransfected luciferase reporter containing five Gal4 binding sites. The activity of Gal4(1-147) was defined as 1.0. Results are the averages ± standard deviations of three independent determinations.

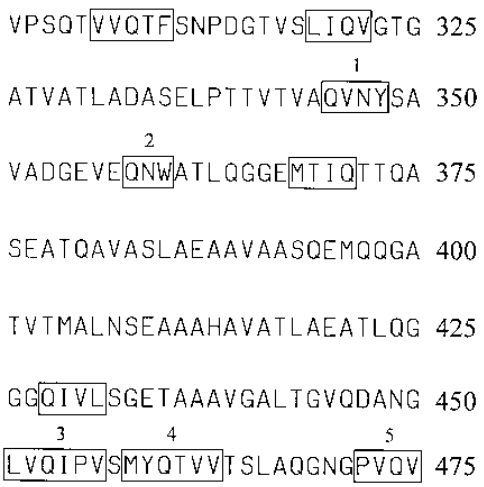

FIG. 5. Amino acid sequence of the NRF-1 transcriptional activation domain. The amino acid sequence of the carboxy-terminal segment of NRF-1 found by deletion mapping to contain the transcriptional activation domain is shown. The Gln-containing hydrophobic clusters are boxed, and those subjected to Ala substitution mutagenesis as described in the legend to Fig. 6 are numbered above.

ther carboxy-terminal deletions to residues 419 and 380 had negligible effects on activation. Although the 338-to-380 construct had no activity by itself, removal of this region from a larger segment comprising residues 338 to 477 to give the 387-to-477 construct resulted in an about fivefold reduction. As with the larger segments, carboxy-terminal deletion of the 387-to-477 construct to residue 419 resulted in a dramatic loss of activity.

The contribution of the region between 338 and 431 was confirmed by amino-terminal deletion of the 338-to-503 construct to residue 431, which resulted in a fivefold reduction in activity. This result confirms that the drop in activity observed upon deletion of amino acids 330 to 380 (Fig. 3) is not strictly due to the lower expression of this fusion protein. The results also suggest that the NRF-1 activation domain has a bipartite structure. A diffuse determinant maps to residues 304 to 380, whereas a second, more concisely defined determinant maps to residues 449 to 477. The major contribution by the former is made by residues 338 to 380. This region apparently requires additional upstream sequences to function independently, as demonstrated by the significant level of activity retained in a larger context upon carboxy-terminal deletion of residues 477 to 419 (Fig. 1 and 3). These upstream determinants account for the significant drop in activity upon amino-terminal deletion of residues 304 to 338 (Fig. 4). Taken together, the results from deletion mapping suggest that the activation domain is composed of distinct functional motifs which are required for maximal transactivation.

Activation by glutamine-containing hydrophobic repeats revealed by alanine substitution mutagenesis. The NRF-1 activation domain does not have a preponderance of amino acids such as glutamines, isoleucines, or prolines that typify other well-characterized activation domains. However, one feature common to the identified regions is that they contain clusters of residues with large hydrophobic side chains with a single glutamine within each cluster (Fig. 5). To determine whether these clusters are responsible for activation, five were selected for alanine substitution mutagenesis. Clusters were mutated in the context of the 338-to-477 fusion because this appeared to be the minimal region which retained the majority of the activity. In three of these, both hydrophobic and glutamine residues were targeted for mutagenesis (Fig. 6). All of the mutated molecules were remarkably consistent in their levels of expression, as revealed by the DNA binding activities recovered from cellular extracts (individual variation of less than 7% from the mean activity [data not shown]), indicating that any observed effects of the mutations were on activation and not on stability or DNA binding. Alanine substitutions for hydrophobic residues either had no significant effect (Fig. 6, mut1Hy and mut5Hy) or reduced activity from three- to fivefold (mut2Hy, mut3Hy, and mut4Hy). The effect on activation was a function of the cluster mutated rather than the number of residues substituted. For example, alanine substitution of a single tryptophan in cluster 2 (mut2Hy) had the same fivefold effect on activation as substitution for three hydrophobic residues in cluster 4 (mut4Hy). Multiple alanine substitutions for hydrophobic residues in clusters 1 (mut1Hy) and 5 (mut5Hy) had no effect on activation. When the alanine-substituted hydrophobic clusters were introduced in combinations of two, the loss of the ability to transactivate was approximately additive. The largest effect of approximately 20-fold resulted from a combination of mutated clusters 2 and 4 (mut2Hy, 4Hy) which individually gave the largest reductions (about 5-fold). Likewise, when the mutation in cluster 2 was combined with those in cluster 5, which alone had no effect on activity, the combined mutant (mut2Hy, 5Hy) was similar in its activity to the cluster 2 mutant (mut2Hy) alone.

A glutamine-rich region coincides with the activation domain of transcription factor Sp1 (8). To determine whether the glutamines within the NRF-1 hydrophobic repeats are required for activation, alanine substitution for glutamine was performed on three of the clusters that displayed a high (mut4Hy), a moderate (mut3Hy), or no (mut5Hy) effect upon substitution of hydrophobic residues. In each case, the mutated molecules were identical to the wild-type protein in their ability to activate the reporter. To determine whether hydrophobic residues can account for the contribution of residues 304 to 338, either the glutamine or the hydrophobic residues within the largest cluster in the interval 306-Val-Val-Gln-Thr-Phe-310 (Fig. 5) were mutated. Again, mutation of the glutamine had no effect whereas mutation of the hydrophobic residues reduced activity by about 20% (not shown). These results demonstrate that the hydrophobic residues in specific clusters contribute greatly to transactivation whereas the glutamines are largely inconsequential. The major contributors to activity are Trp-360 in cluster 2 and the hydrophobic residues in clusters 3 and 4.

The transcription activation domain of NRF-2 is also associated with repeated clusters of hydrophobic residues. The activation domain of nuclear respiratory factor 2 had previously been localized to a single distinct region common to the β and γ subunits and bounded by residues 258 and 327 (19). This region was noted to have repeated hydrophobic clusters containing glutamine. Here, alanine substitution mutagenesis is used to evaluate the contribution of these clusters and to identify essential residues. As with the NRF-1 activation domain, alanine substitution for glutamines within each of the four clusters had little or no effect on the ability of the fusion protein to activate the reporter (Fig. 7, mut1Q, mut2Q, mut3Q, and mut4Q). Likewise, replacement of two of the hydrophobic valines in cluster 1 had no significant effect on activation (mut1Hy). In contrast, replacement of hydrophobic residues within clusters 2 (mut2Hy) and 3 (mut3Hy) dramatically reduced activity approximately 10-fold. Alanine replacement of hydrophobic residues within cluster 4 (mut4Hy) gave a significant but less pronounced reduction. Thus, the hydro-

Gal4/NRF-1	345 1	358 2	451 3	458 4	472 5	Relative Activity
338-477	⊢QVNY—	QNW—	LVQIPV —	MYQTVV —	PVQV⊣	100
Mut 1Hy	⊢QANA—	QNW—	LVQIPV —	MYQTVV —	PVQV⊣	80.1 ± 20.0
Mut 2Hy	⊢QVNY—	QNA—	LVQIPV —	MYQTVV —	PVQV⊣	21.6 ± 6.9
Mut 3Hy	⊢QVNY—	QNW—	LAQAPA —	MYQTVV —	PVQV⊣	42.3 ± 4.0
Mut 3Q	⊢QVNY—	QNW—	LVAIPV —	MYQTVV —	PVQV⊣	99.1 ± 14.7
Mut 4Hy	⊢QVNY—	QNW—	LVQIPV —	MAQTAA —	PVQV⊣	17.6 ± 2.0
Mut 4Q	⊢QVNY—	QNW—	LVQIPV —	MYATVV —	PVQV⊣	94.1 ± 22.0
Mut 5Hy	⊢QVNY—	QNW—	LVQIPV —	MYQTVV —	PAQA⊣	109.3 ± 21.7
Mut 5Q	⊢QVNY—	QNW—	LVQIPV —	MYQTVV —	PVAV⊣	118.3 ± 15.0
Mut 2Hy, 3Hy	⊢QVNY—	QNA —	LAQAPA —	MYQTVV —	PVQV⊣	11.2 ± 3.0
Mut 2Hy, 4Hy	⊢QVNY—	QNA —	LVQIPV —	MAQTAA —	PVQV⊣	4.8 ± 0.8
Mut 2Hy, 5Hy	⊢QVNY—	QNA —	LVQIPV —	MYQTVV —	PAQA⊣	34.4 ± 8.3

FIG. 6. Alanine substitution mutagenesis of the NRF-1 activation domain. Gal4/NRF-1(338-477) fusion protein expression constructs with the indicated mutations were tested for transactivation of a cotransfected luciferase reporter containing five Gal4 binding sites. The alanine substitutions for either glutamine (Q) or hydrophobic residues (Hy) within each cluster (numbered above) are underlined. The activity of the wild-type Gal4/NRF-1(338-477) construct was defined as 100. Results are the averages ± standard deviations of three independent determinations.

phobic residues within clusters 2, 3, and 4 appear to be the major determinants of activity.

These results were confirmed with pairwise combinations of mutated clusters. When clusters with mutated glutamine residues were combined, a modest reduction in activation was observed (mut1Q, 3Q; mut1Q, 4Q; mut2Q, 3Q; mut2Q, 4Q). In contrast, several pairwise combinations of clusters with mutated hydrophobic residues reduced activity to near background levels (mut1Hy, 3Hy; mut2Hy, 3Hy; mut2Hy, 4Hy). The exception was the mut1Hy, 4Hy construct, which had mutations in the least active clusters and retained about 12% of the wild-type activity. Nearly equal amounts of DNA binding activity were present in extracts from transfected cells expressing each fusion protein, indicating that the mutations affected activation and not stability or DNA binding (not shown). Thus, although NRF-2 and NRF-1 are different molecules with respect to DNA binding specificities and subunit composition, they both rely on repeated hydrophobic clusters associated with glutamine residues to activate transcription.

DISCUSSION

Contribution of NRF-1, NRF-2, and Sp1 to respiratory gene expression. Many nuclear genes required for mitochondrial respiratory function rely upon NRF-1 and/or NRF-2 for maximal expression (31). Systematic characterization of a subset of these promoters, from the mtTFA, COXVb, and COXVIIaL genes, has revealed that NRF-1 and NRF-2 in combination with Sp1 are the major transactivators. Recognition sites for all three factors reside in GC-rich regions that are in close proximity to the transcription start site. Mutational analyses have suggested that NRF-1 interacts with other factors in maximizing the activities of these promoters. The presence of a functional NRF-1 site in the mtTFA promoter is required to detect the effects of mutations in either NRF-2 or Sp1 sites (37). This result is consistent with the observation that the formation of properly initiated mtTFA transcripts in an in vitro transcription assay is completely dependent on an intact NRF-1 recognition site. Likewise, mutational analysis of the COXVIIaL promoter indicates that interactions between NRF-1 and upstream Sp1 sites make a significant contribution to its activity (32). Thus, these relatively simple promoters rely upon interactions among a small number of closely associated transcription factors. This arrangement offers the possibility of understanding in molecular detail how structural motifs within these factors contribute to their activation of respiratory chain expression.

Structural motifs that contribute to the function of both Sp1 and NRF-2 (GABP) have been characterized. DNA binding by Sp1 requires carboxy-terminal zinc fingers, while its activation function is localized to functionally redundant regions in the amino-terminal two-thirds of the protein (22). The high glutamine content of these Sp1 A and B domains led to their classification as glutamine-rich activation domains. NRF-2 and its mouse homolog, GABP, are multisubunit transactivators capable of forming heteromeric proteins with distinct DNA binding affinities. Structural domains required for DNA binding as well as homo- and heterodimerization have been delineated for GABP (11, 24, 34) and are highly conserved in NRF-2 (19, 38). The α subunit has an ETS domain that is required both for DNA binding and for its interactions with non-DNA-binding subunits. These auxiliary subunits (β_1 and β_2 in GABP; β_1, β_2, γ_1, and γ_2 in NRF-2) contain ankyrin repeats required for heterodimerization with the α subunit and may or may not contain a carboxy-terminal homodimerization domain. The presence of the latter contributes to high-affinity binding to tandem recognition sites in viral and cellular promoters (11, 24, 34). This may be important in modulating the expression of several cytochrome oxidase subunit promoters

Gal4/NRF-2β_1	261 1 270 2 295 3 305 4	Relative Activity
258-327	⊢IQQVV—QQVITIV—QPIIV—QQVLTV⊣	100
Mut 1Q	⊢IAAVV—QQVITIV—QPIIV—QQVLTV⊣	85.7 ± 12.7
Mut 1Hy	⊢IQQAA—QQVITIV—QPIIV—QQVLTV⊣	85.2 ± 7.1
Mut 2Q	⊢IQQVV—AAVITIV—QPIIV—QQVLTV⊣	85.6 ± 11.5
Mut 2Hy	⊢IQQVV—QQVATAV—QPIIV—QQVLTV⊣	10.7 ± 1.7
Mut 3Q	⊢IQQVV—QQVITIV—APIIV—QQVLTV⊣	107.0 ± 28.4
Mut 3Hy	⊢IQQVV—QQVITIV—QPAAA—QQVLTV⊣	7.7 ± 1.2
Mut 4Q	⊢IQQVV—QQVITIV—QPIIV—AAVLTV⊣	89.4 ± 15.8
Mut 4Hy	⊢IQQVV—QQVITIV—QPIIV—QQALTA⊣	36.3 ± 10.4
Mut 1Q, 3Q	⊢IAAVV—QQVITIV—APIIV—QQVLTV⊣	74.6 ± 10.0
Mut 1Q, 4Q	⊢IAAVV—QQVITIV—QPIIV—AAVLTV⊣	72.8 ± 6.3
Mut 1Hy, 3Hy	⊢IQQAA—QQVITIV—QPAAA—QQVLTV⊣	3.0 ± 0.9
Mut 1Hy, 4Hy	⊢IQQAA—QQVITIV—QPIIV—QQALTA⊣	11.6 ± 2.3
Mut 2Q, 3Q	⊢IQQVV—AAVITIV—APIIV—QQVLTV⊣	76.8 ± 7.2
Mut 2Q, 4Q	⊢IQQVV—AAVITIV—QPIIV—AAVLTV⊣	66.4 ± 4.7
Mut 2Hy, 3Hy	⊢IQQVV—QQVATAV—QPAAA—QQVLTV⊣	1.8 ± 0.6
Mut 2Hy,4Hy	⊢IQQVV—QQVATAV—QPIIV—QQALTA⊣	2.4 ± 0.4

FIG. 7. Alanine substitution mutagenesis of the NRF-2β_1 activation domain. Gal4/NRF-2β_1(258-327) fusion protein expression constructs with the indicated mutations were tested for transcriptional activation of a cotransfected luciferase reporter containing five Gal4 binding sites. Alanine substitutions for either glutamine (Q) or hydrophobic residues (Hy) within each cluster (numbered above) are underlined. The activity of the wild-type Gal4/NRF-2β_1(258-327) construct was defined as 100. Results are the averages ± standard deviations of three independent determinations. Under the conditions used, the relative activity of Gal4(1-147) was 1.3 ± 0.2.

which are characterized by tandemly repeated NRF-2 sites. The domain required for transactivation by NRF-2 has been assigned to a region near the carboxy terminus that is identical in the non-DNA-binding β and γ subunits (19). In contrast to Sp1 and NRF-2, only the DNA binding domain of NRF-1 and related family members has been localized (20, 35).

A complex NLS in NRF-1. In addition to the DNA binding domain, two additional regions of NRF-1 are found here to be necessary for efficient transactivation of a reporter in transfected cells. A highly conserved sequence upstream from the amino-terminal boundary of the DNA binding domain is essential for proper nuclear localization. This conserved domain contains the clusters of basic amino acids that are typical of NLSs.

NLSs have been categorized as either simple or split depending upon whether they exist as a single cluster of basic residues or whether two such clusters separated by 10 or 11 amino acids are required (3). The NRF-1 NLS appears to have properties in common with both types. A simple NLS is characterized by a helix-breaking Gly or Pro at its amino-terminal end and an internal Pro (3). The first basic cluster in NRF-1 (Fig. 2, residues 89 to 93), which was shown to disrupt nuclear localization, has Gly at its amino-terminal end. If the His at residue 95 is included, the extended cluster (88-Gly-Lys-Lys-Arg-Lys-Arg-Pro-His-95) has a Pro as its penultimate residue. However, like the split NLSs, the Lys-Lys-Arg-Lys-Arg motif (residues 89 to 93) is separated from a Lys-Arg-Lys motif (residues 104 to 106) by 10 amino acids. Removal of both of these clusters either by deletion to 109 or point mutation, although highly disruptive, does not completely eliminate nuclear staining. A third cluster (113 to 116) appears to display partial function in the absence of the other two. This would account for the fact that deletion beyond residue 109 is necessary to completely eliminate nuclear staining. The data are consistent with the interpretation that the nuclear localization of NRF-1 requires a bipartite signal (encompassing residues 89 to 106) working in combination with a third basic cluster (113 to 116). This would explain why mutations in both (NLS-mut1,3) are required to eliminate nuclear staining to the same extent as the 1-to-144 deletion.

The results presented here are consistent with recent findings with chicken NRF-1 deletion mutants (15). Nuclear localization of the chicken protein was not affected by removal of either 49 or 79 amino-terminal residues. Surprisingly, deletion to residue 127 or to 172 completely abolished both nuclear and cytoplasmic staining. Given the complete loss of a detectable signal, it remained ambiguous whether protein stability, nuclear localization, or both were affected in these constructs. No loss of signal was observed with any of the mutated constructs described here, and the 1-to-144 deletion was clearly cytoplasmic. The high degree of sequence conservation throughout this region is consistent with it having the same essential function in all members of the NRF-1 family.

The NRF-1 transcriptional activation domain. The three NRF-1 family members display a high degree of sequence divergence in the carboxy-terminal region downstream from the highly conserved DNA binding domain (35). Here, we find this region to be essential for maximal transactivation of reporter promoters in transfected cells. Results obtained with two different activator-reporter systems led to the identification of the same subdomains within a larger region required for activation. In one system, full-length NRF-1 and its deleted derivatives were used to activate a reporter containing four tandem cytochrome *c* NRF-1 sites upstream from a truncated cytochrome *c* promoter. To maximize sensitivity of the assay, NRF-1 deletions were also expressed as Gal4/NRF-1 fusion proteins whose activities were monitored by activation of a reporter containing five tandem Gal4 binding sites upstream from a minimal adenovirus E1b promoter. In each system, deletions to the same endpoints gave nearly identical effects on relative activity. In addition, both the full-length domain (1 to 503) and the largest activation domain deletion (1 to 304) were tested for their ability to transactivate an NRF-1 site in the context of the mtTFA promoter. The results showed that removal of the activation domain identified with the artificial reporter promoters also abolished transactivation of a natural NRF-1-dependent promoter (not shown). Thus, the assignment of activation domain boundaries was independent of the reporter promoter construct and was the same whether analyzed in the context of NRF-1 or as a Gal4 fusion protein.

The functional subdomains, represented schematically in Fig. 8, were further localized by deletion fine mapping. None was enriched in amino acid residues such as Gln, Pro, or Ile used as predominant residues in the classification of activation domains. Although NRF-1 is an acidic protein with a predicted isoelectric point of 4.71, the 304-to-380 subdomain had an Asp-plus-Glu composition that was the same as that of the protein as a whole whereas the carboxy-terminal 449-to-477 subdomain had no acidic residues. The composition of acidic residues in the former was only marginally higher than that of the intervening 380-to-449 region, which had no effect on activity. Thus, the ability of these subregions to contribute to the transactivation of the reporter did not correlate with their acidity or with an unusual abundance of a particular amino acid residue. However, as summarized in Fig. 8, the functional motifs are predominantly hydrophobic, according to several hydrophobicity indices. This differs from VP16, an acidic activator, in which the activation domain coincides with a predicted hydrophilic α-helical structure (10).

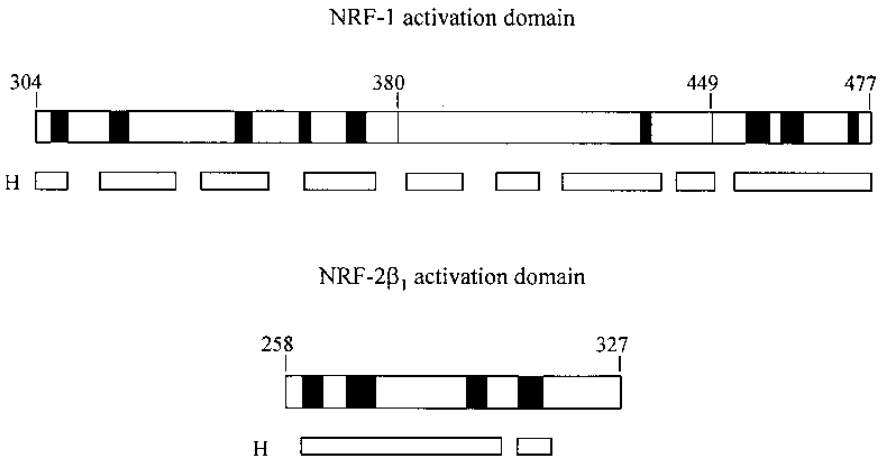

FIG. 8. Schematic summary of NRF-1 and NRF-2 activation domains. Depicted are linear representations (open boxes) of the regions of NRF-1 and NRF-2β_1 that are required for activation of a reporter promoter construct in transfected cells. The amino acid positions relative to the amino-terminal methionine of each protein are indicated above. Clustered glutamine-containing hydrophobic motifs are indicated (closed boxes) within each domain. Depicted below (shaded boxes) are areas of hydrophobicity (H) (21).

Hydrophobic residues have been recently found to be important in both Sp1 and VP16 activation domains. These domains were initially classified as Gln rich and acidic, respectively. The NRF-1 activation domain appears to be a composite of the essential features observed in both VP16 and Sp1. The NRF-1 304-to-380 subregion resembles the VP16 activation domain in that it is highly acidic and requires certain hydrophobic residues for maximal function. A large reduction in NRF-1 activity came from substitution of Ala for Trp-360. This is reminiscent of VP16, in which Phe-442 was found to make a significant contribution (10, 28). Mutation of neighboring hydrophobic residues in NRF-1 had a more modest effect on activity. Alanine substitution at either of two clusters of hydrophobic residues (Val-346 and Tyr-348 or Val-305, Val-306, and Phe-310) reduced activity by about 20%. In addition, in VP16 an alternating pattern of key hydrophobic residues interspersed with acidic residues was observed (10). An imperfect alignment of these hydrophobic residues with those found in other activation domains was proposed (10). None of the functional subregions of NRF-1 were found to conform to this pattern.

Unlike the 304-to-380 subregion, the 449-to-477 subregion of NRF-1 contains no acidic residues but has similar glutamine-containing hydrophobic clusters that are essential. The striking differences in the prevalence of acidic residues in different subregions of the NRF-1 activation domain despite their equivalent functions indicate that acidity per se is not a key factor. The Gln-rich Sp1 B domain is also not very acidic and comprises essential hydrophobic residues. However, although the glutamine content between 449 and 477 (14.3%) is somewhat elevated relative to that of the protein as a whole (8.2%) or the inactive region between 380 and 449 (9.9%), it is considerably lower than that found in Sp1 (9). Also, unlike Sp1, the NRF-1 activation domain does not have an alternating pattern of hydrophobics and glutamines but rather has distinct clusters of hydrophobic residues, each with a single glutamine. Replacement of these glutamines with alanine has no discernible effect on activation. Likewise, the substitution of a number of glutamine residues in the Sp1 B domain did not affect its interaction with the $dTAF_{II}110$ coactivator (14). Thus, although the NRF-1 activation domain has features in common with activation domains in both VP16 and Sp1, the most functionally significant common structural theme lies in the importance of key hydrophobic residues for transcriptional activation.

Comparison of NRF-1 and NRF-2 activation domains. The dependence of many respiratory promoters on NRF-1, NRF-2, or both prompted a comparison of their activation domains. The NRF-1 activation domain resides on the same polypeptide chain as its DNA binding domain, whereas that for NRF-2 is on a separate subunit that heterodimerizes with the DNA-binding ETS domain subunit (19). Nevertheless, the activation domains of the two proteins are similar in several respects. They are both present in acidic proteins embedded in regions of high overall acidity. In addition, they are both in regions of high hydrophobicity. Finally, their activities are both highly dependent on discrete glutamine-containing clusters of hydrophobic residues in which the glutamines make little or no functional contribution. It should be noted that the analysis presented here leaves open the possibility that other residues that are not in the identified clusters are functionally significant. The data also do not resolve whether the clustered arrangement is necessary or whether key residues within each cluster are the major contributors.

The most striking difference between the two activation domains lies in the arrangement of the hydrophobic motifs. In NRF-2, the activation domain is restricted to a relatively compact region of the molecule, with the major determinants residing in a segment of about 41 amino acids (residues 270 to 310). In contrast, in NRF-1, the functional determinants span approximately 40% of the molecule (residues 304 to 477). Within this region, functionally distinct subdomains are separated by an inactive segment that is rich in alanine (29.6% between residues 380 and 449). This arrangement may offer a degree of flexibility in interactions with the initiation complex that may help facilitate expression in certain promoter contexts or under different physiological conditions. It is interesting to note that the NRF-2 β and γ subunits that contain the activation domain differ in their ability to homodimerize and thereby confer high-affinity binding to tandem sites (34, 38). Consequently, in the presence of the tandem recognition sites that exist in several cytochrome oxidase promoters, multiple copies of the NRF-2 activation domain would be bound to the promoter and stabilized through protein-protein contacts. There-

fore, homodimerization of β subunits may create an activation surface that is similar to that achieved by the bipartite arrangement of subdomains within the single NRF-1 polypeptide chain. It will be of interest to determine whether the similarities in activation domain structure contribute to the observed interactions among these transcription factors in the expression of genes required for mitochondrial respiratory function.

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