Serine Phosphorylation within a Concise Amino-terminal Domain in Nuclear Respiratory Factor 1 Enhances DNA Binding*

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Mitochondrial respiratory function requires the expression of essential gene products from both nuclear and mitochondrial genetic systems. Because of its compact structure and limited coding capacity, the mitochondrial genome encodes only 13 proteins along with the tRNAs and mRNAs required for their translation (for review, see Ref. 2). All of these proteins are subunits of the inner membrane respiratory complexes. Thus, nuclear genes must specify the majority of respiratory subunits and all of the proteins required for the expression, maintenance, and replication of mitochondrial DNA (for review, see Ref. 8). One approach to understanding nucleo-mitochondrial interactions in mammalian cells is to identify the nuclear transcription factors that govern the expression of these genes.

NRF-1 is originally identified as a nuclear transcription factor that acts on mammalian genes encoding cytochrome c and a number of other respiratory proteins (4, 6, 10). A possible role for the factor in intergenicomic communication is supported by the discovery of functional NRF-1 recognition sites in nuclear genes specifying the rate-limiting heme biosynthetic enzyme, 5-aminolevulinate synthase (14), and components of the mitochondrial transcription and replication machinery (6, 18, 22). The latter include the RNA subunit of mitochondrial RNA processing endonuclease, an enzyme implicated in the formation of mtDNA replication primers and mtTFA, an activator of mitochondrial transcription (for review, see Refs. 8 and 16). These findings led to a model whereby NRF-1, along with other transcription factors, helps coordinate the synthesis and function of respiratory proteins from both genomes (6, 18, 22). In addition, it has been postulated that NRF-1 may play a role in other developmental and growth regulatory processes (10, 17).

NRF-1 has been purified and a cDNA clone isolated and characterized (10, 17). The protein is related through a novel DNA binding domain to developmental regulatory proteins from sea urchins (12) and Drosophila (13). This highly conserved DNA binding domain is in the NH2-terminal half of the molecule, and the conservation extends beyond its NH2-terminal boundary through a region containing a complex nuclear localization signal (17, 23). A large COOH-terminal domain in NRF-1 is required for transcriptional activation and is not conserved in the lower eukaryotic proteins (23). Here, we establish that a concise NH2-terminal domain in NRF-1 serves as a target for phosphorylation in vivo and in vitro. The phosphorylation occurs on multiple serine residues and enhances DNA binding activity. Although the phosphodomain overlaps a region required for NRF-1 homodimerization, phosphorylation does not alter the monomer-dimer equilibrium in the absence of DNA. Rather, phosphorylation appears to cause an intrinsic change in the NRF-1 dimer thereby enhancing its DNA binding.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The following adaptors were used for epitope tagging in plasmid constructions.

AD1 GTACCAATCATACCATACCACTACACTCAGAG
GTTGATGTTAGTTGATGACTCTAG

AD2 GTACCAATCCCTAGCGAACCCCGATTACCCTGAG
GTTAGGAGATCTCAGGAGCTGACACTCCTAG

AD3 GTAGAGTTACCATATCCTTATGATGTACCTAGCT
TCCATGTATAGCAGAATCTACACAGGACTATGGCAGCATG

Untagged versions of pSG5NRF-1 expression vectors have been described previously (17, 23). Adaptor AD1 was used to generate 6xHis-tagged NRF-1. This adaptor encodes 6 histidine residues and a TGA stop codon between overhangs for Asp718I and BamHI restriction sites, which were used to clone it into pGEM7Zf(+) (Promega) to derive 7Z-6xHis. An EcoRI-MscI fragment from pSG5NRF-1 (17) and an MscI-Asp718I fragment from Gal4/NRF-1(138–503) (23), from which the

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The abbreviations used are: NRF-1, nuclear respiratory factor 1; HA, hemagglutinin; CKII, casein kinase II; SRF, serum response factor; MEF2C, myocyte enhancer factor 2C; PAGE, polyacrylamide gel electrophoresis.
natural NRF-1 termination codon had been previously removed, were cloned into the EcoRI and Asp718I sites of 7Z-6xHis. This provided an EcoRI-BamHI fragment in which the COOH-terminal coding region of NRF-1 was fused in-frame with 6 histidine residues encoded by the AD1 adaptor. This fragment, along with an NcoI-EcoRI fragment from pS5GNRF-1, which was used to clone it into pET3d, gave 7Z-6xHis-NRF-1. The NcoI and BamHI sites of pET3d (15) to yield the expression vector pET3d-NRF-1–6xHis.

NRF-1 tagged with the HA epitope was generated in a similar fashion using adaptor AD2. AD2 encodes the HA epitope (YYPDVPDYA) and a TGA stop codon between Asp718I and BamHI restriction sites, which were inserted into pET3d-NRF-1–6xHis. This led to the replacement of the COOH-terminal coding region of NRF-1 in pS5GNRF-1 with the same region including the HA tag to give the expression vector pSG5NRF-1–HA. To produce the 3xHA-tagged version, the adaptor AD3 was used. This has an XhoI overhang, an Asp718I restriction site, the coding sequences for the HA tag, and an Asp718I overhang, which, upon cloning into the same site, no longer matches the recognition sequence for that enzyme. This adaptor was cloned into the XhoI and Asp718I sites upstream of the HA tag and BamHI site in 7Z-HA. This gave a fragment containing a 2xHA tag (7Z-2xHA) (as encoded by the original Asp718I site) and a new Asp718I site created at the 5' end of the double tag. 7Z-2xHA was cut with XhoI and Asp718I (just as with 7Z-HA) and another copy of AD3 cloned within those sites to give 7Z-3xHA. This again destroyed the old Asp718I site and created a new one at the 5' end of the triple tag. The 3xHA-tag was removed from 7Z-3xHA by Asp718I digestion and used to replace the simple tag encoded within the same restriction sites in pS5GNRF-1–HA to give pS5GNRF-1–3xHA.

The NH2-terminal deletion mutants, Mut 1 and Mut 2, were made by polymerase chain reaction. The forward primer NFR-1S242 (5'-GAAGGATCCTGAGGAAGACGCGCCTTCT-3') or NFR-1S263 (5'-GAAGGATCCTGAGGAAGACGCGCCTTCT-3') was used with the reverse primer NFR-1-932A (5'-ATGCAAGAACGGAATTTTTACAC-3'). Numbers are relative to the first nucleotide of the original NRF-1 cDNA (17). NFR-1S242 and S263 also contain a translation initiating ATG within the HA-tag, and an Asp718I restriction site. The polymerase chain reaction products were digested with NcoI and PstI (a natural site in the NRF-1 coding region), and these fragments were cloned into the same sites in the pET3d expression vectors (9). This led to the replacement of the COOH-terminal coding region with the AD1 fragment from pET3d-NRF-1–6xHis (which provided the NRF-1 COOH-terminal codon and used to replace the single tag encoded within the same restriction sites). The NRF-1NH2-terminal region, was ligated into the NcoI and BamHI sites of pET3d (15) to yield the expression vector pET3d-NRF-1–6xHis.

In Vitro Phosphorylation—Recombinant NRF-1 (used in HeLa cell transfections and in vivo labeling) was partially purified by (NH4)2SO4 precipitation as described (9). This partially purified fraction was purified further by Ni2+-nitrilotriacetae-agarose chromatography (Qiagen) as recommended by the manufacturer. Recombinant proteins eluted from the Ni2+-column were dialyzed against 25 m M Tris-HCl, pH 7.5, 0.5 m M EDTA, 0.1% SDS, and 10% glycerol. Recombinant proteins were concentrated by centrifugal ultrafiltration (Centricon), and stored at −70 °C.

Cell Culture and in Vivo Labeling—Conditions for culture of COS-1 and HeLa cells have been described previously (20). COS-1 cells were transfected by calcium phosphate precipitation as described (5, 7, 20). HeLa cells were transfected in six-well plates using LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer. For in vivo labeling, transfected HeLa cells were rinsed and then refed with 650 μCi of phosphate-free culture medium. 200 μCi of [32P]orthophosphate (Amersham Corp.) was added, and cells were grown for 4 h before harvesting for immunoprecipitation. For labeling of endogenous NRF-1, HeLa cells were grown to 90% confluence in a 100-mm dish, rinsed, and refed with 3 ml of phosphate-free medium. [32P]Orthophosphate (1.5 mCi) was then added, and cells were grown for 4 h before harvesting for immunoprecipitation.

Immunoprecipitation—All procedures were carried out at 4 °C unless otherwise noted. HeLa cells growing on dishes were washed twice with cold phosphate-buffered saline and lysed for 30 min in situ with RIPA buffer (50 m M Tris-HCl, pH 8.0, 150 m M NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS) containing 5 m M NaF, 500 n M okadaic acid, 1 μg/ml benzamidine, 1 μg/ml aprotinin, 1 m M phenylmethylsulfonyl fluoride, and complete protease inhibitors. The lysate was spun at 10,000 × g for 10 min to pellet insoluble material. The supernatant was incubated for 1 h with 2 μl of rabbit anti-NRF-1 antiserum (or preimmune antiserum) preadsorbed with 1% bovine liver acetone powder (Sigma) and 0.3% bovine serum albumin. RIPA buffer (50 μl) containing 10% protein A-agarose beads (Boehringer Mannheim) was added and the mixture incubated with gentle rocking for 1 h. The immune complexes were collected by centrifugation at 10,000 × g for 1 min and washed three times with RIPA buffer. The samples were resuspended in SDS sample buffer, heated to 85 °C for 10 min, and loaded onto 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon (Millipore) membranes as described below before performing autoradiography.

Immunoblotting—Proteins were transferred to Immobilon membranes (Millipore) using a Bio-Rad Trans-Blot apparatus as recommended by the manufacturer. Membranes were blocked overnight at 4 °C in TBST (20 m M Tris-HCl, pH 7.5, 500 m M NaCl, 0.05% Tween 20) containing 5% non-fat dry milk. For detection of non-epitope-tagged NRF-1 in extracts of cells in COS-1 or HeLa cell transfections, membranes were incubated with rabbit anti-NRF-1 antiserum at 1:2,000 dilution in TBST containing 5% non-fat dry milk for 1 h at room temperature. After washing in TBST, membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham) at 1:5,000 dilution in TBST containing 5% non-fat dry milk for 1 h at room temperature. After incubation with the secondary antibody, membranes were washed 6× in TBST and proteins detected with an enhanced chemiluminescence kit (Amersham) as recommended by the manufacturer. For membranes with HA-tagged NRF-1 and mutants (used in HeLa cell transfections and in vivo labeling) the salt concentration in TBST was reduced to 50 m M. These proteins were detected with the 12CA5 monoclonal antibody (Boehringer Mannheim) at 1:10,000 dilution followed by horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Sigma) at 1:5,000 dilution.

In Vitro Phosphorylation—Recombinant NRF-1 and derivatives were phosphorylated with recombinant CKII (New England Biolabs) at 30 °C in reaction buffer containing 20 m M Tris-HCl, pH 7.5, 50 m M KCl, 10 m M MgCl2, 2 m M dithiothreitol, 200 μM ATP, and 1 μg of substrate protein. Reactions were performed under identical conditions without ATP for controls. To calculate the stoichiometry of phosphorylate added per NRF-1 molecule, a small amount of [γ-32P]ATP was included. A portion of the labeled proteins was precipitated with cold 15% trichloroacetic acid. The precipitated samples were trapped on MF-Millipore membrane filters and washed with 10% trichloroacetic acid. The filters were placed in scintillant and incorporation determined by scintillation counting. The values obtained were corrected for background by the method of Kunkel (3). The mutated fragment was then used to replace the equivalent region in pS5GNRF-1–3xHA or pET3d-NRF-1–6xHis to make expression vectors with the desired alanine substitutions.

Purification of Recombinant Proteins—pET3d expression vectors constructed as described above were transformed into E. coli BL21(DE3) pLysS (15). Bacterial cultures were induced, and overexpressed recombinant protein was partially purified by (NH4)2SO4 precipitation as described (9). This partially purified fraction was purified further by Ni2+-nitrilotriacetate-agarose chromatography (Qiagen) as recommended by the manufacturer. Recombinant proteins eluted from the Ni2+-column were dialyzed against 25 m M Tris-HCl, pH 7.5, 0.5 m M EDTA, 0.1% SDS, and 10% glycerol. Recombinant proteins were concentrated by centrifugal ultrafiltration (Centricon), and stored at −70 °C.
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Tris-HCl, pH 7.9, 0.5 mM EDTA, 50 mM KCl, 0.5 mg/ml bovine serum albumin, 10% (v/v) glycerol, 0.5 mM dithiothreitol and stored at −70 °C until needed.

**Phosphoamino Acid and Phosphopeptide Analysis—**Proteins phosphorylated in vitro or immunoprecipitated from labeled cells were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Immobilon (for phosphoamino acid analysis) or nitrocellulose (for phosphopeptide analysis) as described above. Labeled proteins were visualized by autoradiography. For phosphoamino acid analysis, bands excised from Immobilon membranes were hydrolyzed directly in 5.7 N HCl (24). Phosphoamino acids were analyzed by two-dimensional electrophoresis at pH 1.9 and 3.5 on thin layer cellulose plates (25, 26) using the Hunter thin layer electrophoresis system (CBS Scientific) as recommended by the manufacturer. For phosphopeptide analysis, labeled bands excised from nitrocellulose membranes were digested in situ overnight at 37 °C with 50 ng of trypsin or chymotrypsin as described (11). Peptides obtained from digestion were mixed with an equal volume of 2 × SDS sample buffer, boiled, and loaded onto a 10% SDS-polyacrylamide gel. The gel was dried and phosphopeptides visualized by autoradiography.

**Mobility Shift Assays—**Oligonucleotides used for mobility shift assay in this study have been described previously and represent NRF-1 binding sites from the rat somatic cytochrome c (RC4), rat cytochrome c oxidase subunit VIc-2 (COX VIc), tyrosine aminotransferase (TAT), and human mitochondrial transcription factor A (mtTFA) genes (1, 4, 6, 10, 18). Nuclear extracts were prepared from transfected COS-1 cells as described elsewhere (19). Mobility shift assays were performed with these extracts as described previously (7, 10). Mobility shift assays with recombinant NRF-1 and mutants were performed in 20 μl of buffer containing 25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl, 10% (v/v) glycerol, 5 μg of casein, 100 ng of sonicated calf thymus DNA, 200 ng of recombinant protein, and 10 fmol of oligonucleotide that was 32P end labeled using the Klenow fragment of DNA polymerase I. Mobility shift reactions were incubated at room temperature for 15 min before electrophoresis on native 5% polyacrylamide gels (acylamide:bis-acrylamide, 58:1) in 25 mM Tris-HCl, pH 8, 33.5 mM boric acid, 0.5 mM EDTA. Gels were then dried and DNA-protein complexes visualized by autoradiography. For quantification of the difference in binding activity of phospho and non-phospho forms of NRF-1, 500 pg of recombinant protein was used with no calf thymus DNA and varying amounts (0.1 fmol–1 pmol) of oligonucleotide. The signal from bound and free oligonucleotide was quantified using a Molecular Dynamics PSI PhosphorImager.

**RESULTS**

**NRF-1 Is Phosphorylated on Serine Residues in Vivo—**Examination of the sequence of NRF-1 revealed a number of potential phosphorylation sites. To determine whether NRF-1 is a phosophoprotein in vivo, the protein was immunoprecipitated from 32P-labeled HeLa cell extracts following in vivo labeling. A single intensely labeled band migrating at the expected 68 kDa was detected upon gel electrophoresis of immunoprecipitates under denaturing conditions (Fig. 1A, lane 2). No signal was obtained with preimmune serum (lane 1). To confirm that the precipitated protein is NRF-1, the experiment was repeated using cells transfected with a NRF-1 expression vector or the same vector lacking the NRF-1 coding region as a control. A labeled 68-kDa band was markedly enhanced in precipitates from cells transfected with the NRF-1 expression vector (lane 1A, lane 4) relative to those transfected with the control vector (lane 3) under conditions optimized for minimal background from the endogenous protein. Thus, the NRF-1 protein expressed from both endogenous and transfected genes is phosphorylated in vivo.

It was of interest to determine whether the protein expressed from the transgene is phosphorylated on the same sites as the endogenous NRF-1. To this end, the 32P-labeled NRF-1 proteins that had been transferred to membranes after gel electrophoresis were subjected to proteolytic digestion. Trypsin cleavage yielded a major 18-kDa labeled peptide for both endogenous and transfected proteins (Fig. 1B, lanes 1 and 2), whereas chymotrypsin cleavage generated a more complex pattern of peptides which was also identical between two protein samples (lanes 3 and 4). Phosphoamino acid analysis was performed on endogenous and transfected proteins to determine whether the same amino acid residues were phosphorylated. In both cases, phosphoserine was the predominant phosphoamino acid detected along with trace amounts of phosphothreonine (Fig. 1C). Thus, phosphorylation of the protein expressed from the transgene is indistinguishable from that of the endogenous NRF-1 protein in vivo.

**NRF-1 Phosphorylation Is Confined to Distinct Serine Residues within a Concise NH2-terminal Domain—**A computer search revealed several consensus phosphorylation sites for known kinases within the NH2-terminal end of NRF-1. To identify potential phosphorylation sites, several NH2-terminal deletions (Fig. 2A) were expressed from transgenes, and their ability to act as substrates for phosphorylation was assayed by immunoprecipitation of labeled extracts. The transgenes were constructed so that one copy of the HA epitope is incorporated to the COOH terminus of the expressed proteins. This allows the expressed protein to be detected with a specific monoclonal antibody directed against the tag. A significant reduction in phosphorylation was detected upon removal of serine and threonine residues up to and including serine 44 (Fig. 2B, Mut 1). Removal of the remaining serine and threonine residues in the NH2-terminal domain by deletion to residue 78 (Mut 3) resulted in a minor increase in signal. Immunoblotting of the same samples using the anti-HA antibody shows that the wild-type and mutated proteins are present in equivalent amounts.
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FIG. 2. Deletion mapping of the NRF-1 phosphorylation domain in vivo. A, schematic of the NH2-terminal region of wild-type (WT) NRF-1, the deletion mutants Mut 1, Mut 2, Mut 3, and the alanine substitution mutants Mut 8xA and Mut 12xA. The positions of potential serine and threonine phosphorylation sites at which alanine substitution mutants were made are indicated above. Mut 1 contains a deletion of residues 1–41 as well as an alanine substitution for serine 44. B, HeLa cells were transiently transfected with an expression vector encoding HA-tagged versions of wild-type NRF-1 (lane 1) or the indicated mutant (lanes 2–4). After labeling with [32P]orthophosphate, NRF-1 or mutants were immunoprecipitated with anti-NRF-1 serum and analyzed by SDS-PAGE. Proteins were transferred to Immobilon membranes before autoradiography. The arrow marks the position of endogenous NRF-1. C, proteins transferred to membranes in B were detected using an anti-HA-tag monoclonal antibody (12CA5). Molecular mass standards are indicated at the left.

Thus, the differences observed do not result from differences in the expression or precipitation efficiencies among the constructs. Nearly identical results were obtained upon expression of the same deletion series in COS cells with the exception that Mut 2 gives a somewhat stronger phosphorylation signal in COS than in HeLa cells (not shown). These results, along with the identification of phosphoserine as the predominant phosphoamino acid, demonstrate that major in vivo phosphorylation sites are localized to serines 36–61.

The precise identity of the phosphorylated residues was determined by converting all 12 potential serine and threonine phosphorylation sites within the NH2-terminal domain to alanines by site-directed mutagenesis (Fig. 2A, Mut 12xA). These include all 8 serines between residues 1 and 78 and the 4 threonines (7, 31, 51, and 62) which best match consensus phosphorylation sites. Each residue was restored individually to wild-type in expression constructs, and the ability of each site to be phosphorylated was tested by transfection and immunoprecipitation. The transgenes for these proteins were constructed such that three copies of the HA epitope are incorporated at the COOH terminus of the expressed proteins. This allows the expressed protein to be resolved from the endogenous protein and detected with a specific monoclonal antibody directed against the tag. Elimination of all 12 residues in Mut 12xA almost completely abolished the phosphorylation of NRF-1 expressed from the transgene (Fig. 3A, lane 1). This confirms the results obtained with NH2-terminal deletions (Fig. 2) and establishes that the major phosphorylation sites reside within a concise NH2-terminal domain encompassing residues 7–62. Restoration of each of the potential sites individually shows that serines 39, 44, and 46 are the major phosphorylation sites (Fig. 3A, lanes 5, 6, and 7), whereas serines 47 and 52 (lanes 8 and 10) may be minor sites. Immunoblotting using antibodies directed against the HA epitope that is specific for transfected proteins confirms that differences in transfection efficiency, expression, or immunoprecipitation by themselves are insufficient to account for the differences in phosphorylation signal (Fig. 3B). These results are largely consistent with the those obtained with the NH2-terminal deletions. The one apparent discrepancy with the deletion results is that serine 52 is at least partially phosphorylated (Fig. 3A, lane 10). However, overexpression of Mut 2 in COS cells yields a detectable signal (not shown) which likely results from phosphorylation of this residue. In addition, serine 47 appears to be well phosphorylated in COS cells. This may result from higher levels of expression of the transgene or from a cell type-specific difference.

Phosphorylation of NRF-1 by CKII in Vitro Occurs at the Sites Utilized in Vivo and Stimulates DNA Binding—The region of NRF-1 phosphorylation does not coincide with previously defined domains required for DNA binding, nuclear localization, or transcriptional activation (17, 23). To assay the potential functional consequences of phosphorylation it is useful to mimic the in vivo phosphorylation pattern in an in vitro system. Because several of the sites resemble the consensus for phosphorylation by CKII (35), this enzyme was tested using purified recombinant NRF-1 proteins prepared from the wild-type and various mutated derivatives of NRF-1 as substrates. The results closely parallel those obtained with the proteins expressed in transfected cells. It was estimated that CKII can incorporate approximately 5 pmol of phosphate/pmol of wild-type NRF-1 under optimum conditions (Fig. 4A, lane 1). This was reduced to about 3 pmol in Mut 1, 2 pmol in Mut 2, and 0.2 pmol in Mut 3 (Fig. 4A, lanes 2–4), indicating that the enzyme acts on the same NH2-terminal domain phosphorylated in vivo.

The major discrepancy between the in vivo and in vitro results is that Mut 2 is phosphorylated to a significant degree in vitro but not in vivo. Phosphoamino acid analysis shows that phosphoserine is preferentially lost upon progressive NH2-terminal deletion resulting in equal amounts of phosphoserine and phosphothreonine in Mut 2 (Fig. 4B). This, along with the stoichiometry of phosphorylation in Mut 2 (2 pmol of phosphate incorporated per pmol of NRF-1), indicates that the additional phosphorylation observed using CKII in vitro results from the phosphorylation of a serine and a threonine residue between...
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**Fig. 4.** Phosphorylation of NRF-1 by CKII in vitro. A. 6xHis-tagged versions of wild-type NRF-1 or mutated derivatives described schematically in Fig. 2 were overexpressed in bacteria. Purified recombinant proteins were incubated with CKII and [γ-32P]ATP and analyzed by SDS-PAGE. Molecular mass standards are indicated at the left. Proteins were transferred to Immobilon membranes before autoradiography. B. Proteins phosphorylated in A were hydrolyzed with 5.7 N HCl and analyzed for phosphoamino acid content as described under "Experimental Procedures." Electrophoresis in the horizontal direction was at pH 1.9 and in the vertical direction at pH 3.5 with the origin at the lower right. Arrows mark the positions of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) standards.

Amino acids 49 and 78. This is confirmed by the finding that mutation of all 8 serine residues in the entire NH2-terminal domain reduced the molar incorporation of phosphate to about 1 pmol (Mut 8xA, lane 5) of phosphothreonine (Fig. 4B, Mut 8xA) which was reduced to background levels in Mut 12xA (lane 6). These observations are consistent with the conclusion that CKII phosphorylates the same serines that are phosphorylated in vivo. However, the enzyme is also able to phosphorylate at least 1 serine and 1 threonine residue between amino acids 51 and 62. This is consistent with the phosphorylation of serine 52 detected above (Fig. 3A) as well as the phosphorylation of Mut 2 in COS cells (not shown).

One attractive hypothesis is that phosphorylation may modulate NRF-1 function by affecting its ability to bind DNA. To examine this possibility, the DNA binding activities of the phospho- and non-phospho forms of the wild-type and various mutant proteins were assayed. The results show a marked stimulation of the NRF-1 DNA binding activity by phosphorylation (Fig. 5A, lanes 1 and 2). To quantify this stimulation, 500 pg of phospho or non-phospho forms of NRF-1 were incubated with varying amounts (0.1 fmol–1 pmol) of NRF-1-binding oligonucleotide. The results indicate that at saturation, ~12-fold more phosphate than non-phospho protein is bound (not shown). This stimulation is reduced in Mut 1 (lanes 3 and 4) and is largely eliminated in Mut 2 (lanes 5 and 6), which removes the major serine phosphorylation sites utilized in vivo and in Mut 3 (lanes 7 and 8), which is not phosphorylated. The importance of serine phosphorylation is demonstrated by the absence of a stimulatory effect on DNA binding in Mut 8xA (lanes 9 and 10), which retains the threonine phosphorylation site. As with Mut 3, Mut 12xA, which is not phosphorylated, is also not stimulated in binding. The enhancement of NRF-1 DNA binding was observed using known recognition sites from several target genes including cytochrome c (Fig. 5A, lanes 1 and 2), cytochrome oxidase subunit Vlc-2 (COX6c, lanes 1–4), tyrosine aminotransferase (TAT, lanes 5–8), and mitochondrial transcription factor A (mtTFA, lanes 9–12) genes by electrophoretic mobility shift assay.

**Phosphorylation Stimulates NRF-1 DNA Binding without Affecting Dimer Formation**—The NRF-1 consensus binding site is a palindrome consisting of tandemly repeated GC motifs (6, 17) suggesting that the protein binds DNA as a homodimer. If this is the case, phosphorylation may stimulate DNA binding by facilitating subunit interactions. To determine whether a dimer is the DNA-binding species, a series of transgenes expressing truncated derivatives of NRF-1 was constructed. Both COOH-terminal and NH2-terminal deletions were expressed in transfected cells, and nuclear extracts were assayed for DNA binding to the cytochrome c NRF-1 site. COOH-terminal deletions to residues 419 (Fig. 6, construct B) and 304 (construct C) and NH2-terminal deletions to residues 78 (construct E) and 110 (construct F) all retained DNA binding activity similar to that of the full-length wild-type protein (construct A) (Fig. 6A, lanes 1–3 and 6 and 7). The various deletion mutants were also expressed at levels similar to wild-type as measured by immu-
Fig. 6. Binding of NRF-1 to DNA as a dimer. A, COS-1 cells were mock transfected (lane 1) or transfected with plasmids expressing wild-type NRF-1 or deleted derivatives either singly (lanes 2–7) or paired with the wild-type (lanes 8–12) as indicated. A schematic of the expressed proteins labeled alphabetically is shown below. Nuclear extracts made from transfected cells were tested for binding to the NRF-1 site in the rat somatic cytochrome c gene by electrophoretic mobility shift assay. B, nuclear extracts from transfected cells were analyzed by SDS-PAGE and immunoblotting with anti-NRF-1 serum. Molecular mass standards are indicated at the left.

Fig. 7. Dimerization by phosphorylated and nonphosphorylated NRF-1. A, recombinant wild-type NRF-1 or the indicated mutated derivatives produced in bacteria were incubated with CKII in the absence (lanes 1–5) or presence (lanes 6–10) of ATP. Dimer formation was analyzed by native PAGE and the proteins visualized by staining with Fast Stain (Zoion Research). B, proteins incubated with CKII in A were assayed for binding to the NRF-1 site in the rat somatic cytochrome c gene by electrophoretic mobility shift assay.

When treated with CKII in the absence of ATP, the wild-type protein was entirely dimeric (lane 1), whereas Mut 3 was almost entirely monomeric (lane 4). The intermediate deletions, Mut 1 and Mut 2, migrate as mixtures of monomer and dimer (lanes 2 and 3). The same results were obtained in the presence of ATP but the absence of the enzyme (not shown). Thus, an NH2-terminal domain between residues 1 and 78 is required for NRF-1 to maintain its homodimeric structure in the absence of DNA. This domain overlaps with all of the major sites of serine phosphorylation.

To assay potential effects of phosphorylation on dimer formation, the wild-type and mutated proteins were treated with CKII in the presence of ATP. Under these conditions, phosphorylation had no effect on the proportions of monomer and dimer detected on native gels (Fig. 7A, lanes 6–9). As expected, however, inclusion of ATP increased the DNA binding activities of those molecules containing the major serine phosphorylation sites (Fig. 7B, lanes 6 and 7). Mut 12xA in which all of the serine and threonine phosphorylation sites are converted to alanines is indistinguishable in its migration from wild-type in either the presence or absence of ATP (Fig. 7A, lanes 5 and 10), indicating that phosphorylation of these residues is not essential for dimer formation. Therefore, phosphorylation stimulates DNA binding without promoting dimer formation in the absence of DNA.

**DISCUSSION**

The preponderance of evidence points to a role for NRF-1 in nucleo-mitochondrial interactions in mammalian cells (6, 17, 18). Functional NRF-1 sites are present in the majority of nuclear genes encoding respiratory subunits and also in several genes required for mitochondrial transcription, replication, and heme biosynthesis (22). In addition, NRF-1 may serve a more
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integrative role in coordinate gene expression by acting on target genes encoding key components in the pathways of cellular metabolism, signal transduction, and chromosome maintenance (10, 17). Several structural domains in NRF-1 which are required for its biological function have been resolved. A DNA binding domain within the NH$_2$-terminal two-thirds of the molecule is highly conserved in a small family of eukaryotic regulatory proteins which includes the sea urchin P3A2 factor and the Drosophila erect wing gene product (17). These proteins are required for proper neuromuscular development in their respective organisms. The COOH-terminal domain is not conserved among family members. In NRF-1, this domain contains clusters of hydrophobic residues that are required for transcriptional activation (23). In addition to the DNA binding domain, significant sequence conservation with P3A2 is present between residues 37 and 116. Part of the sequence conservation in this region (residues 88–116) has been ascribed to a complex nuclear localization signal located just upstream from the DNA binding domain (21, 23). No other functions had been assigned to the remainder of this conserved region.

Here, we establish that NRF-1 is a phosphoprotein and that in vivo phosphorylation occurs within a concise NH$_2$-terminal domain on serine residues 39, 44, 46, 47, and 52. Four of the five serines (39, 44, 46, and 47) are conserved in P3A2, but it is unknown whether they are phosphorylated in sea urchins. All five serines conform to the consensus for phosphorylation by CKII (35) (S/T-X-X-acidic, where the acidic residue can be glutamate, aspartate, phosphoserine, or phosphotyrosine), and the purified enzyme phosphorylates these sites in vitro. All five are surrounded by acidic amino acids that are prevalent in known CKII sites, and all but serine 44 have the requisite acidic residue at the +3 position. In the serine 44 site it is likely that the negative charge at this position is contributed by phosphoserine 47. Although CKII is the most likely candidate for the in vivo NRF-1 kinase, the in vivo and in vitro phosphorylation patterns are not identical. The major difference is that the region between residues 48 and 77 is more intensely phosphorylated in vitro than it is in vivo. The stoichiometry of phosphorylation suggests that this region accepts two phosphates on a molar basis. In addition to serine 52, which is weakly phosphorylated in vivo, threonines 51 and 62 conform to the consensus. These residues can account for the in vitro threonine phosphorylation detected in wild-type and mutated proteins. Thus, it is likely that these residues are more accessible to phosphorylation in the in vitro CKII reaction. It is important to note, however, that threonine phosphorylation by itself does not contribute to enhanced DNA binding. Only that majority of sites which are phosphorylated both in vivo and in vitro can account for the stimulatory effect of phosphorylation on NRF-1 DNA binding activity.

The formation of NRF-1 heterodimers in transfected cells expressing both wild-type and deleted proteins constitutes compelling evidence that the protein binds DNA as a dimer. This is consistent with the dyad symmetry in the pallidomeric NRF-1 recognition site (6, 17). Although glutaraldehyde cross-linking results in two species migrating as monomer and dimer on denaturing gels (not shown), attempts to form heterodimers in vitro by mixing native proteins were unsuccessful. This has also been observed with the chicken homolog of NRF-1 and has been attributed to a low equilibrium dissociation constant for the homodimer (21). Synthesis of wild-type and deleted proteins in the same cellular compartment in transfected cells allows free association among subunits and results in the detection of heterodimers.

Here, we demonstrate that the stability of the NRF-1 dimer in the absence of DNA is disrupted upon progressive NH$_2$-terminal deletion to residue 78. Upon deletion to this residue, the predominant species detected on native gels is clearly monomer. This indicates that a strong determinant of dimerization is located in the NH$_2$-terminal region of the protein apart from the DNA binding domain. However, a protein with the 1–78 deletion binds DNA as a homodimer, can heterodimerize with wild-type on DNA (Fig. 6), and can be cross-linked to a dimer with glutaraldehyde (not shown). We note that the Mut 3 protein does appear to bind DNA less strongly than the wild-type, which is consistent with removal of a dimerization domain. This is in agreement with the results obtained with chicken NRF-1 which suggest an overlap between DNA binding and dimerization functions between residues 79 and 172 (21).

We conclude that the NH$_2$-terminal domain identified here is essential for stable subunit interactions in the absence of DNA. In addition, determinants within the DNA binding domain contribute a level of stability sufficient for DNA binding and for detection with cross-linking agents. However, we find no evidence that phosphorylation stimulates DNA binding by promoting dimer stability. Complete elimination of all NH$_2$-terminal phosphorylation sites by site-directed mutagenesis does not result in any detectable dissociation to monomer on native gels. This suggests that phosphorylation affects DNA binding at the level of DNA-protein interaction.

One might expect that the phosphorylation-dependent enhancement of DNA binding would result in an increased ability of wild-type NRF-1 to activate transcription compared with Mut 3. We have previously compared wild-type NRF-1 and Mut 3 for their ability to trans-activate a luciferase reporter whose expression was driven by four copies of the NRF-1 binding site from the rat cytochrome c gene cloned upstream of a minimal promoter in transient cotransfection experiments in COS-1 cells (23). In those experiments, both proteins activated transcription by about 7–8-fold over that of an empty vector. The absence of a difference between these constructs under conditions of transient transfection most likely results from the fact that the transgene expresses NRF-1 or Mut 3 at very high levels. Since Mut 3 can still bind DNA and has an intact activation domain, overexpression may compensate for its reduced DNA binding activity relative to that of the wild-type phosphorylated protein.

Several transcription factors are now known to be phosphorylated at CKII consensus sites both in vivo and in vitro. In particular, NRF-1 shares a number of interesting similarities with two such factors, SRF and MEF2C. Both SRF (30–32, 36) and MEF2C (27) resemble NRF-1 in that phosphorylation of a restricted number of serine residues adjacent to the region of the molecule which contacts DNA accounts for enhanced DNA binding. In SRF, dephosphorylation of specific serine residues resulted in decreases in both its association and dissociation rates leading to the conclusion that phosphorylation does not alter the equilibrium binding affinity for DNA (31, 32). We also note that phosphorylation results in a marked increase in the rate of dissociation of NRF-1 from DNA, suggesting that its exchange rate on DNA may be similarly affected. Conversion of the essential serines to acidic amino acids in both SRF and MEF2C had the same stimulatory effect on DNA binding as phosphorylation. In addition, phosphorylation does not alter the ability of SRF to dimerize (33) or of MEF2C to interact with other myogenic transcription factors (27). In both cases it has been postulated that introduction of a negative charge may induce a conformational change that affects the DNA binding domain (27, 33).

S. Gugneja and R. C. Scarpulla, unpublished observations.
Such a mechanism is consistent with the finding that NRF-1 phosphorylation also stimulates DNA binding without altering the association between homologous subunits. These similarities suggest that SRF, MEF2C, and NRF-1 may be targets for the same or very similar kinase-mediated signaling pathway.

Control of NRF-1 function by phosphorylation is an attractive mechanism given the proposed role for the factor in nucleo-mitochondrial interactions and the integration of other growth regulatory pathways. Although CKII activity is stimulated by serum growth factors (for review, see Ref. 34) there is conflicting evidence that this stimulation leads to the activation of gene expression through the modification of specific transcription factors. No change in the phosphorylated state of SRF or the relative amounts of SRF isoforms has been found in response to growth factor treatment. Stable expression of a mutant SRF that binds its recognition site at constitutively high levels had no effect on c-fos expression (30). By contrast, phosphorylation of the CKII sites in the upstream binding factor markedly elevates the trans-activation of their ribosomal RNA target genes. In addition, the phosphorylated state of upstream binding factor but not its level of expression, is stimulated in proliferating relative to serum arrested cells, and this correlates with enhanced ribosomal RNA transcription (28, 29). One unexplored possibility is that phosphorylation of transcription factors such as NRF-1 by CKII or related kinases occurs in response to intracellular signals that communicate the respiratory state of the mitochondria to the nuclear transcriptional apparatus. Such a mechanism may link the expression of nuclear genes to the availability of respiratory energy. The results presented here represent an essential first step in understanding the potential role of phosphorylation in NRF-1-dependent gene expression.

REFERENCES
1. Evans, M. J., and Scarpulla, R. C. (1988) Mol. Cell. Biol. 8, 35–41
2. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333
3. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
4. Evans, M. J., and Scarpulla, R. C. (1989) J. Biol. Chem. 264, 14361–14368
5. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456–467
6. Evans, M. J., and Scarpulla, R. C. (1990) Genes Dev. 4, 1023–1034
7. Virbasius, J. V., and Scarpulla, R. C. (1991) Mol. Cell. Biol. 11, 5631–5638
8. Clayton, D. A. (1991) Annu. Rev. Cell Biol. 7, 453–478
9. Pagone, P., Kato, H., Sumimoto, H., Kretzschmar, M., and Roeder, R. G. (1991) Nucleic Acids Res. 19, 6650
10. Chau, C. A., Evans, M. J., and Scarpulla, R. C. (1992) J. Biol. Chem. 267, 6999–7006
11. Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E., and Kent, S. B. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6970–6974
12. Calzone, F. J., Hong, C., Teplow, D. B., Cutting, A. E., Zeller, R. W., Britten, R. J., and Davidson, E. H. (1991) Development 112, 335–350
13. Demise, S. M., and White, K. (1995) Mol. Cell. Biol. 15, 3641–3949
14. Brautigan, L. I., and May, B. K. (1997) J. Biol. Chem. 261, 1109–1117
15. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
16. Shadel, G. S., and Clayton, D. A. (1993) J. Biol. Chem. 268, 16083–16086
17. Virbasius, C. A., Virbasius, J. V., and Scarpulla, R. C. (1993) Genes Dev. 17, 2431–2445
18. Virbasius, J. V., and Scarpulla, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1309–1313
19. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
20. Gopalakrishnan, L., and Scarpulla, R. C. (1995) J. Biol. Chem. 270, 18019–18025
21. Gomez-Cuadrado, A., Martin, M., Noël, M., and Ruiz-Carrillo, A. (1995) Mol. Cell. Biol. 15, 6670–6685
22. Scarpulla, R. C. (1996) Trends Cardiovasc. Med. 6, 39–45
23. Gugneja, S., Virbasius, C. A., and Scarpulla, R. C. (1996) Mol. Cell. Biol. 16, 5708–5718
24. Kamps, M. P., and Sefton, B. M. (1989) Anal. Biochem. 176, 22–27
25. Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) Methods Enzymol. 99, 387–402
26. Hunter, T., and Sefton, B. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
27. Molkentin, J. D., Li, L., and Olson, E. N. (1996) J. Biol. Chem. 271, 17199–17204
28. Voï, R., Schnapp, A., Kuhn, A., Rosenbauer, H., Hirschmann, P., Stunnenberg, H. G., and Grunert, I. (1992) EMBO J. 11, 2211–2218
29. O’Mahony, D. J., Xie, W., Smith, S. D., Singer, H. A., and Rothblum, L. I. (1992) J. Biol. Chem. 267, 35–38
30. Manak, J. R., and Prywes, R. (1993) Oncogene 8, 703–711
31. Janknecht, R., Hipskind, R. A., Houthaev, T., Nordheim, A., and Stunnenberg, H. G. (1992) EMBO J. 11, 1045–1054
32. Marais, R. M., Hasun, J. J., McGuigan, C., Wynne, J., and Treisman, R. (1992) EMBO J. 11, 97–105
33. Manak, J. R., and Prywes, R. (1991) Mol. Cell. Biol. 11, 3652–3659
34. Litchfield, D. W., and Luscher, B. (1993) Mol. Cell. Biochem. 127/128, 187–199
35. Songyang, Z., Lu, K. P., Kwon, Y. T., Teai, L. H., Filhel, O., Cochet, C., Brickley, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hoekstra, M. F., Bienis, J., Hunter, T., and Cantley, L. C. (1996) Mol. Cell. Biol. 16, 6486–6493
36. Manak, J. R., de Bisschop, N., Kris, R. M., and Prywes, R. (1990) Genes Dev. 4, 955–967