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Sequential Serum-dependent Activation of CREB and NRF-1 Leads to Enhanced Mitochondrial Respiration through the Induction of Cytochrome *c**

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Progression through the cell cycle requires ATP for protein synthesis, cytoskeletal rearrangement, chromatin remodeling, and protein degradation. The mechanisms by which mammalian cells increase respiratory capacity and ATP production in preparation for cell division are largely unexplored. Here, we demonstrate that serum induction of cytochrome *c* mRNA and processed protein in quiescent BALB/3T3 fibroblasts is associated with a marked increase in mitochondrial respiration. Cytochrome *c* was induced in the absence of any increase in citrate synthase activity or in subunit IV of the cytochrome *c* oxidase complex mRNA or protein, indicating that the enhanced respiratory rate did not require a general increase in mitochondrial biogenesis or respiratory chain expression. Transfections with a series of cytochrome *c* promoter mutants showed that both nuclear respiratory factor 1 (NRF-1) and cAMP-response element-binding protein (CREB) binding sites contributed equally to induced expression by serum. Moreover, CREB and NRF-1 were phosphorylated sequentially in response to serum, and the NRF-1 phosphorylation was accompanied by an increase in its ability to *trans*-activate target gene expression. The results demonstrate that the differential transcriptional expression of cytochrome *c*, through sequential transcription factor phosphorylations, leads to enhanced mitochondrial respiratory capacity upon serum-induced entry to the cell cycle.

Entry into the cell cycle requires energy in the form of ATP for the execution of a number of regulatory and metabolic events. Protein synthesis consumes large amounts of cellular energy and is required for entry into S phase (1). In quiescent 3T3 fibroblasts, cellular protein content increases within 4–5 h of serum stimulation and precedes DNA replication by several hours (2). Nontoxic inhibition of mitochondrial respiratory function inhibits progression to G1 in parallel with a reduction in cellular ATP (3). Protein synthesis (1) and degradation (4) as well as the depolymerization of the microtubular network during interphase (5) all depend upon the intracellular ATP concentration. ATP has also been implicated in the regulation of cyclin-dependent kinases, which in turn control cell proliferation (6).

The bulk of cellular ATP comes from the mitochondrial electron transport chain and oxidative phosphorylation system (7). This system comprises of five integral membrane complexes along with the dissociable electron carriers, ubiquinone and cytochrome *c*. An electrochemical gradient of protons is established across the mitochondrial inner membrane as a result of electron transfer. The dissipation of this proton gradient through the ATP synthase (complex V) is coupled to the synthesis of ATP (Fig. 1) (8). The respiratory apparatus is the product of ~90 different genes localized in both the nuclear and mitochondrial genomes (9, 10). Although the mitochondrial genes are essential, they encode only 13 respiratory polypeptides along with the tRNAs and rRNAs required for their translation in the mitochondrial matrix. Thus, nuclear genes specify the majority of respiratory subunits, and nuclear gene products govern mitochondrial gene expression in part by controlling the transcription and replication of mitochondrial DNA (11–14).

In mammalian cells, little is known of the signaling pathways that mediate changes in the expression and function of the respiratory apparatus in meeting cellular energy demands. One means for responding to changing requirements for ATP is to modulate the synthesis or activity of respiratory chain constituents in response to extracellular signals. For example, in yeast, metabolites in the environment regulate the expression of cytochrome *c* and other respiratory proteins through the action of specific transcriptional activators and repressors (15). Cytochrome *c* participates in the reduction of oxygen by cytochrome oxidase, the terminal and putative rate-limiting step of electron transfer (16). The induction of cytochrome *c* by oxygen and nonfermentable carbon sources is part of a shift to aerobic growth. It is conceivable that in mammalian cells, the rate of respiration under certain physiological conditions may also be governed by regulating levels of cytochrome *c*. Mechanisms governing cytochrome *c* expression in mammalian cells may also be important in controlling apoptosis and levels of reactive oxygen species (reviewed in Ref. 17).

We have previously isolated the human and rodent cytochrome *c* genes and investigated the determinants of cytochrome *c* transcription in mammalian cells (13). The somatically expressed gene has a complex promoter comprised of recognition sites for NRF-1,[1] Sp1, CAAT box factors, and CREB/activating transcription factor among others (18). Tandem cAMP-response elements (CREs) were *trans*-activated by CREB in the presence of protein kinase A, and these CREs

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[1] The abbreviations used are: NRF-1, nuclear respiratory factor 1; CREB, cAMP-response element-binding protein; CRE, cAMP-response element; PBS, phosphate-buffered saline; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine diHCl; COX, cytochrome *c* oxidase; COXIV, subunit IV of the cytochrome *c* oxidase complex; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CKII, casein kinase II; RCO4, rat cytochrome oxidase subunit IV gene.

accounted for the cAMP induction of cytochrome *c* mRNA (19). These findings represent the only established link between a signaling molecule (cAMP) and a specific transcriptional activator (CREB) in the expression of a respiratory chain gene in vertebrate systems.

NRF-1 was originally characterized as an activator of cytochrome *c* expression and was subsequently found to act on many nuclear genes required for mitochondrial respiratory function (18, 20). These include genes encoding respiratory subunits (21), the rate-limiting heme biosynthetic enzyme (22), as well as mtDNA transcription and replication factors (20, 23). NRF-1 is phosphorylated both *in vivo* and *in vitro* on serine residues within a concise amino-terminal domain (24). Phosphorylation of these sites *in vitro* enhances NRF-1 DNA binding activity, suggesting that these modifications may regulate NRF-1 function. Such a mechanism might allow the nuclear transcriptional machinery to respond both to extracellular signals and to intracellular ATP concentrations in controlling the expression of the respiratory chain. However, no *in vivo* regulatory function has yet been ascribed to NRF-1 phosphorylation.

Here, we examine changes in respiratory activity and cytochrome *c* gene expression associated with the serum-induced proliferation of quiescent 3T3 fibroblasts. We find that the transcriptional induction of cytochrome *c* is intimately associated with enhanced respiratory activity in preparation for entry to the cell cycle. The results demonstrate that a change in respiratory capacity can be implemented through the differential expression of a key respiratory protein rather than through coordinate synthesis of the entire chain.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/3T3 cells, clone A31, passage 64 from the American Type Culture Collection, were tested for contact inhibition and used between passages 67 and 69 because the respiratory response to serum was diminished or absent in transformed cells. Tissue culture reagents were from Sigma except for fetal bovine serum, which was from HyClone Laboratories, Inc. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and supplemented with 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, and 1 mM sodium pyruvate. For all experiments, cells were plated at a density of 5×10^3 cells/cm^2 in growth medium and allowed to grow for two days until ~40–50% confluent. The cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) and then serum-starved in Dulbecco's modified Eagle's medium, 0.5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin for 60 h. Following starvation, the cells were stimulated in Dulbecco's modified Eagle's medium, 20% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin for the indicated times. The viable cell number was found to be constant over 12 h of serum stimulation. Schneider's *Drosophila* line-2 (SL2) cells were obtained from the American Type Culture Collection at passage 519. All experiments were carried out between passages 521 and 541. Cells were cultured at 25 °C in Schneider's insect medium (Sigma) containing 10% fetal bovine serum as described (25).

Respirometry—Cells assayed for oxygen utilization were harvested using a 1× trypsin/EDTA solution, collected by centrifugation at 1000 × *g,* and resuspended in PBS. Viable cells were counted as determined by dye exclusion using 1× trypan blue solution. The cell suspension was pelleted and resuspended to 5×10^4 cells/μl in respiration buffer containing 30 mM Tris-HCl (pH 7.4), 75 mM sucrose, 50 mM KCl, 0.5 mM EDTA, 0.5 mM $MgCl_2$, and 2 mM potassium phosphate. Oxygen utilization by ~1.5×10^6 cells was measured in 650 μl of air-saturated respiration buffer at 37 °C with constant mixing using an oxygen-sensitive electrode and dual oxygen electrode amplifier (INSTECH Model 203). After the rate of oxygen uptake driven by endogenous cellular substrates was recorded, rates of oxygen utilization were determined upon sequential addition of the following substrates and inhibitors to the indicated final concentrations: 10 mM each glutamate plus malate (pH 7.4), 0.5 μM CCCP, 0.02 μM rotenone, 0.02 μM antimycin A, 2 mM ascorbate plus 0.2 mM TMPD, pH 6.0, 0.3 mM KCN, pH 8.0 (26). The ascorbate/TMPD respiration rate was measured as that which was KCN sensitive. No increase in oxygen uptake was observed in the presence of digitonin indicating that the substrates are permeable.

Enzymatic Assays—Cells were lysed in 20 mM phosphate, pH 7.4, 0.1% laurylmaltoside at 5×10^3 cells/μl and incubated for 15 min on ice. Citrate synthase activity was assayed using extract from 2.5×10^4 cells in a 300-μl reaction containing 100 mM Tris, pH 8.0, 0.5 mM acetyl-CoA, 0.5 mM 5,5′-dithio-bis(2-nitrobenzoic acid). The reaction was started by the addition of oxaloacetate to a final concentration of 0.5 mM. The change in absorbance at 419 nm was followed for 5 min (27). For the cytochrome oxidase (COX) assay, a 2-mM solution of ferricytochrome *c* was reduced with several grains of sodium dithionite and chromatographed on a G50 Sephadex column (0.7 × 10 cm), and the ferricytochrome *c* eluate was assayed at 550 nm. COX activity using extract derived from 2×10^4 cells was assayed in 300 μl of 10 mM phosphate, pH 7.4, 10 μM ferricytochrome *c* by the change in absorbance at 550 nm (28).

Cytochrome Analysis by Difference Spectra—For spectrophotometric determination of cellular cytochrome contents, ~3×10^6 cells were suspended in 20 mM Tris, pH 7.5, 1% Triton X-100, 0.1 mM ferricyanide and incubated on ice for 10 min. The suspension was centrifuged at 10,000 × *g*; the supernatant was divided between reference and sample cuvettes (80-μl quartz). A Cary 300 Bio UV-visible spectrophotometer was zeroed across a spectral range of 420 to 640 nm with both cuvettes in place. Reduction of the sample with ascorbate plus TMPD was carried out in 0.4 mM KCN, 4 mM ascorbate, and 0.4 mM TMPD, and the absorbance spectra taken. The sample was subsequently reduced with 0.4 mM sodium dithionite, and a second absorbance spectra determined. Spectral data were expressed as reduced minus oxidized cytochrome spectral absorbance (29).

Immunoblotting—Nuclear extracts were prepared for immunoblotting as described (30). For preparation of BALB/3T3 whole cell extracts, cells from a 150-mm plate at 60% confluence were lysed in 150 μl of RIPA buffer (50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) containing 5 mM NaF, 500 nM okadaic acid, 1 mM sodium orthovanadate as phosphatase inhibitors, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and allowed to incubate on ice for 30 min. The lysate was cleared by centrifugation at 20,000 × *g* for 5 min, and the supernatant was collected. SL2 whole cell extracts were prepared from 10% of the transfected cells by lysis in 40 μl of RIPA. The remaining cells were used for reporter enzyme assays as described below.

Extracts were subjected to SDS-polyacrylamide gel electrophoresis (36.5 acrylamide:1 bis-acrylamide) at 200 V for detection of cytochrome *c* and COXIV (15% polyacrylamide) and CREB and phosphoCREB (10% polyacrylamide). The gels were transferred to either polyvinylidene difluoride (cytochrome *c* and COXIV) or to nitrocellulose (CREB and NRF-1). COXIV and cytochrome *c* were transferred in 10 mM CAPS, 10% methanol, pH 11. CREB was transferred in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Immunodetection was carried out with ECL reagents according to the manufacturer (Amersham Pharmacia Biotech).

Plasmid Constructions—Plasmid p7ZfmCyt.*c* for the synthesis of a mouse cytochrome *c* riboprobe was made by cloning a 261-base pair *Hin*cII-*Bam*HI fragment from the mouse gene (31) into *Sma*I-*Bam*HI-digested pGEM7Zf. A plasmid for the production of a rat COXIV riboprobe was constructed by cloning the 281-base pair *Bgl*II-*Pvu*II fragment containing exon 4 (32) into the *Bam*HI and *Hin*cII sites of pGEM4Blue. The rat cytochrome *c* promoter luciferase plasmid, pGL3RC4/-326, was constructed by excising the promoter, 5′-untranslated region, and first intron fragment from pRC4CAT/-326 (33) with *Bgl*II, blunting with Klenow DNA polymerase, and digesting with *Xho*I. The resultant fragment was cloned into pGL3 basic vector (Promega) that had been cut with *Hin*dIII, blunt-ended with Klenow, and digested with *Xho*I. pGL3RC4/-66, pGL3RC4/-326, and pGL3RC4/-326;CRE DM1, were all derived from their respective CAT plasmids (19, 33) by substituting a *Xho*I-*Hin*dIII fragment. pGL3RC4/-326 NRF-1Mut was constructed from the parent plasmid pGL3RC4/-326 using oligonucleotide RC4NRF-1MutF 5′-ACCATGCTAGCCCTCATTAGCGCGCACCTTGC-T-3′ and the QuikChange™ site-directed mutagenesis kit (Promega). Similarly, pGL3RC4/-326;CRE DM1/NRF-1Mut was constructed from pGL3RC4/-326;CRE DM1 using RC4NRF-1MutR 5′-AGCAAGGTGCG-CGCTAATGAGGGCTAGCATGGT-3′. The *Hin*dIII-*Xho*I fragments from the amplified reaction products were subcloned back into the parent pGL3RC4/-326 and sequenced. The construction of pSG5NRF-1/3xHA has been described (24). In pPac5c, the *Drosophila* actin 5c promoter drives expression of cloned coding regions (34). To make pPac5cNRF-1, pSG5NRF-1/119-1662 was digested with *Eco*RI and *Pst*I, and the 230-base pair fragment was cloned into pBluescript. The resulting plasmid was digested with *Eco*RV and *Pst*I to release the NRF-1 fragment. Next, pET3dNRF-1/6His was cut with *Pst*I and *Acc*65I and,

TABLE I
Enzyme activities over a time course of serum stimulation

Enzyme/substrate	Serum stimulation (h)		
	0	3	12
	Activity		
	fmol/min/cell		
Endogenous[a,b]	0.88 ± 0.09 (1)[c]	2.20 ± 0.28 (2.0)	3.96 ± 0.12 (4.3)
Glutamate + malate[a,b]	1.65 ± 0.35 (1)	3.75 ± 0.36 (1.8)	5.13 ± 0.32 (2.9)
Ascorbate + TMPD[a,b,d,e]	3.49 ± 0.67 (1)	4.86 ± 0.35 (1.1)	8.02 ± 0.19 (2.2)
Cytochrome oxidase[e,f]	18.33 ± 0.97 (1)	18.01 ± 0.47 (0.8)	10.64 ± 0.19 (0.6)
Citrate synthase[b,f]	3.16 ± 0.28	4.10 ± 0.26	3.38 ± 0.48

[a] fmol of O_2 consumed/min/cell using 1.5×10^6 intact cells in a 650-μl reaction volume.
[b] Activity values are the average of three independent experiments ± the standard error of the mean.
[c] Relative activities normalized to citrate synthase.
[d] Assay performed in the presence of CCCP.
[e] fmol of ferrocytochrome *c* oxidized/min/cell using a total cell extract from 2×10^4 cells in a 300-μl reaction volume.
[f] fmol of citric acid synthesized/min/cell using a total cell extract from 2×10^4 cells in a 300-μl reaction volume.

together with the *Eco*RV/*Pst*I NRF-1 fragment, cloned into pPac5c linearized with *Eco*RV and *Acc*65I. pPac5cNRF-1Mut8xA was made from pSG5NRF-1Mut 8xA (24) as described for pPac5cNRF-1. p4xNRF-1Luc has been described (35). pSG5CREB was made by subcloning the *Eco*RI/*Bgl*II fragment from pRSVCREB into the *Eco*RI/*Bam*HI sites of pSG5.

RNase Protection Assays—Total RNA was prepared using TRIzol reagent (Life Technologies, Inc.), and RNase protections were performed as described (36). Reactions contained 5 μg of total RNA from BALB/3T3 cells and 15 μg of yeast tRNA. Antisense probes for hybridization were prepared by linearizing plasmids with *Mlu*I for mouse cytochrome *c* or *Eco*RI for RCO4 and transcribing with T7 polymerase. The 345-nucleotide cytochrome *c* probe gave a protected fragment of 175 nucleotides, whereas the 350-nucleotide RCO4 probe gave a protected fragment of 132 nucleotides. The protected products were run on a 6% acrylamide (29:1), 8 M urea gel and were visualized by autoradiography.

Transfections—BALB/3T3 cells were plated at 5×10^3 cells/cm^2 in 6-well plates and incubated for 24–36 h. For transfections, 200 ng of pGL3RC4 reporter plasmid, 100 ng of pCMV β-gal (CLONTECH), and 3.7 μg of pGEM7Zf were added to 100 μl of Dulbecco's modified Eagle's medium. SuperFect (Qiagen)(5 μl) was added and vortexed for 10 s, and the mixture was incubated for 10 min. After the addition of 600 μl of growth medium, the suspension was added to a PBS-washed well and incubated for 6–8 h. The cells were washed three times with PBS and then starved with 2 ml of starvation medium for 48 h. The medium was changed to either stimulation or starvation medium, and the cells were incubated for an additional 13.5 h. COS cells were transfected with calcium/phosphate-precipitated DNA (33). SL2 cells were plated at 10^6 cells/ml in 100-mm dishes and transfected with calcium/phosphate-precipitated DNA (25).

Luciferase and β-Galactosidase Assays—Cell extracts were prepared, and luciferase assays were performed using reagents from Pharmingen. Spectrophotometric β-galactosidase assays were performed using the β-galactosidase enzyme assay system (Promega).

Immunoprecipitations and Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (24). BALB/3T3 cells were labeled with [^{32}P]orthophosphate after 60 h in starvation medium. The medium was changed to phosphate-free stimulation media, and 200 μCi of [^{32}P]orthophosphate was added. Cells were incubated for 12 h before harvesting with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). Transfected SL2 cells were labeled with [^{32}P]orthophosphate 24 h after removal of the DNA precipitate. The cells were replated in 4 ml of medium containing 1.5 mCi of [^{32}P]orthophosphate. After 4 h they were washed with PBS and lysed in RIPA buffer.

For *in vivo* ^{35}S-labeling, cells were plated and after 16 h washed with PBS and labeled with 5 ml of methionine-free growth medium containing 250 μCi of [^{35}S]methionine at 1000 Ci/mmol. After 16 h cells were washed with PBS, and 5 ml of methionine-free starvation medium containing 250 μCi of [^{35}S]methionine was added. After 60 h the cells were again washed with PBS, and 5 ml of methionine-free stimulation medium containing 250 μCi of [^{35}S]methionine was added. The cells were incubated for 12 h, washed with PBS, and lysed in RIPA buffer. *In vivo* labeled NRF-1 was immunoprecipitated from BALB/3T3 extracts as described (24). Extracts were precleared by incubation with normal rabbit IgG for 3 h followed by precipitation with protein A-agarose.

RESULTS

Serum Stimulation of BALB/3T3 Fibroblasts Leads to Enhanced Cytochrome c-dependent Respiration—We observed a marked increase in the endogenous rate of respiration in quiescent BALB/3T3 cells that were serum-stimulated (Table I). The increased respiration began within 3 h of stimulation and extended through at least 12 h. The elevated endogenous respiration rate, measured on a per cell basis, coincided with a similar increase in glutamate/malate-dependent respiration in the presence of an uncoupler. This is consistent with the enhancement of electron flow from NADH through the entire electron transport chain (Fig. 1). An uncoupler (CCCP) was included to dissipate the proton gradient and thus alleviate respiratory control through complex V (Fig. 1). The increase in electron transport did not result from a general increase in respiratory chain synthesis or mitochondrial biogenesis. The activity of the mitochondrial matrix enzyme, citrate synthase, which is correlated with mitochondrial content (37), remained unchanged in this time interval (Table I). In addition, there was no increase in cytochrome *c* oxidase activity as measured spectrophotometrically using reduced cytochrome *c* as a substrate. Thus, the increased respiration did not coincide with a general increase in mitochondrial proliferation or respiratory chain expression.

Although cytochrome oxidase activity was unchanged, increased respiration was observed using ascorbate plus TMPD (Table I), substrates that enhance respiration by reducing endogenous cytochrome *c* (Fig. 1). This reaction was performed in the presence of antimycin A, which blocks the reduction of cytochrome *c* by NADH, and respiration was measured as the cyanide-sensitive component of the activity. In this manner, it was possible to dissect out the contribution of cytochrome *c* to the reduction of oxygen. The stimulation of oxygen consumption by ascorbate plus TMPD in response to serum stimulation demonstrated that cytochrome *c*-dependent respiration contributed to the increased oxygen utilization.

Cytochrome c Protein Is Preferentially Induced Relative to COXIV upon Serum Stimulation—The observed increase in cytochrome *c*-dependent respiration may be explained by the serum induction of cytochrome *c* relative to other respiratory components. Because cytochrome oxidase activity was not increased, the level of cytochrome *c* relative to cytochrome oxidase subunit IV (COXIV) was measured by immunoblotting. The results show a time-dependent elevation of cytochrome *c* protein in total cell extracts, under conditions where COXIV protein levels remained unchanged (Fig. 2). When densitometric values were normalized to COXIV, cytochrome *c* increased ~1.5-fold after 3 h of serum stimulation and about 4-fold after

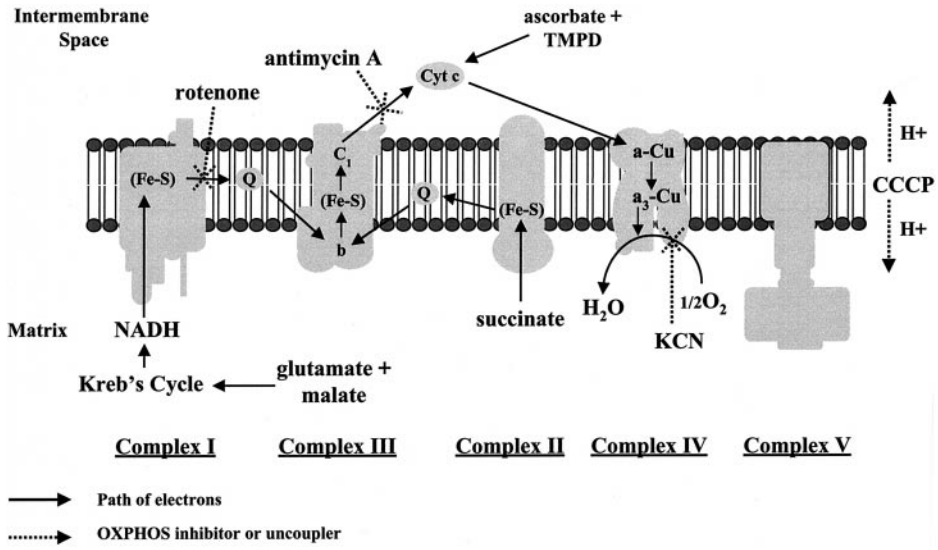

FIG. 1. **Diagrammatic representation of the electron transport chain and oxidative phosphorylation system.** *Shaded* objects imbedded in the lipid bilayer of the mitochondrial inner membrane represent respiratory complexes I–V. Dissociable electron carriers cytochrome *c* (*Cyt c*) and coenzyme Q (*Q*) are also shown. *Arrows* depict the pathway of electron flow and the points of entry of electrons from the various electron donors (glutamate + malate, ascorbate + TMPD, and succinate). *Broken lines* indicate the sites of inhibition for the various respiratory inhibitors (rotenone, antimycin A, KCN, and CCCP) used in the experiments.

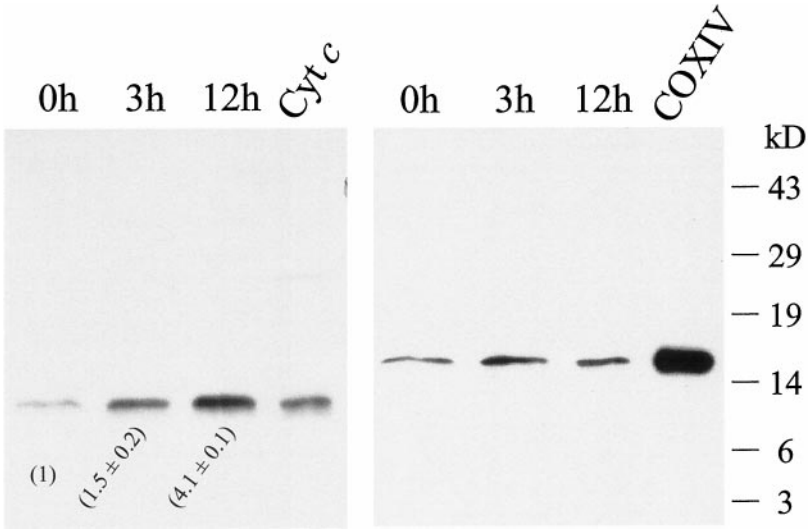

FIG. 2. **Comparison of cytochrome *c* and COXIV protein levels in BALB/3T3 cells over a time course of serum stimulation.** The *left panel* shows the results from an immunoblot using anti-cytochrome *c* serum on whole cell extracts from cells serum-stimulated for 0, 3, or 12 h. The *right panel* shows an identical immunoblot using the same cell extracts except using a monoclonal antibody against COXIV as the primary antibody. The far *right lane* in each immunoblot contains either purified cytochrome *c* or COXIV as a positive control. The molecular masses of protein standards are shown at the right. *Numbers* in parentheses denote the fold induction of cytochrome *c* normalized to that of COXIV ± the standard error of the mean for two independent experiments.

12 h. These values correlate well with the overall increase in respiration observed in these time intervals (Table I).

To determine whether the induction of cytochrome *c* protein leads to higher levels of heme-containing holocytochrome, difference spectra obtained after 0 and 12 h of serum induction were compared. A clear increase in cytochrome $c + c_1$ absorbance at 552 nm was observed after 12 h of serum induction using ascorbate plus TMPD as reducing agents (Fig. 3*A*). These reduce the chain from cytochrome *c* on down and leave cytochrome c_1, which is part of the reductase (complex III), in the oxidized state (Fig. 1). To confirm that cytochrome *c* absorbance is a major contributor to the increased absorbance at 552 nm, additional difference spectra were obtained using dithionite to reduce the entire cytochrome complement. No further increase in absorbance at 552 nm over that measured using ascorbate plus TMPD was observed (Fig. 3*B*). These results indicate that holocytochrome *c* induction is largely responsible for the observed increase in absorbance under these conditions. By contrast to the elevation in absorbance at 552 nm, no serum stimulation of cytochrome $a + a_3$ absorbance at 605 nm using either ascorbate plus TMPD (Fig. 3*A*) or dithionite (Fig. 3*B*) as reducing agents was observed. The *a*-type cytochromes are part of cytochrome oxidase, and thus the results were consistent with the observations that COX activity (Table I) and COXIV protein levels (Fig. 2) were also unchanged in this time interval. The $a + a_3$ absorbance also served as an internal control for the observed change in cytochrome *c* absorbance. The results establish that the differential induction of holocytochrome *c* contributes to the serum-dependent enhancement of mitochondrial respiration.

Functional Recognition Sites for CREB and NRF-1 Mediate the Serum Induction of the Cytochrome c Promoter—It seemed likely that cytochrome *c* induction was mediated by an increase in gene expression. Thus, steady-state levels of cytochrome *c* and COXIV mRNAs were compared over the time course of serum induction. The results demonstrated a marked increase in cytochrome *c* mRNA with no significant change in COXIV mRNA (Fig. 4). Cytochrome *c* mRNA induction began within 3 h of serum stimulation and paralleled the rise in both cytochrome *c* protein and cellular respiration. The relatively rapid kinetics of mRNA induction places the cytochrome *c* gene among the delayed early class of serum-responsive genes (38).

Because many such genes are induced at the transcriptional level, it was of interest to determine whether the cytochrome *c* promoter was serum-responsive. To this end, cytochrome *c* promoter constructs were transfected into BALB/3T3 fibroblasts. Following serum starvation, the cells received fresh medium containing either 0.5 or 20% fetal bovine serum, and promoter activities were determined using extracts derived from starved or stimulated cells. The results demonstrated a significant serum induction of cytochrome *c* promoter activity using a construct containing all of the previously defined *cis*-

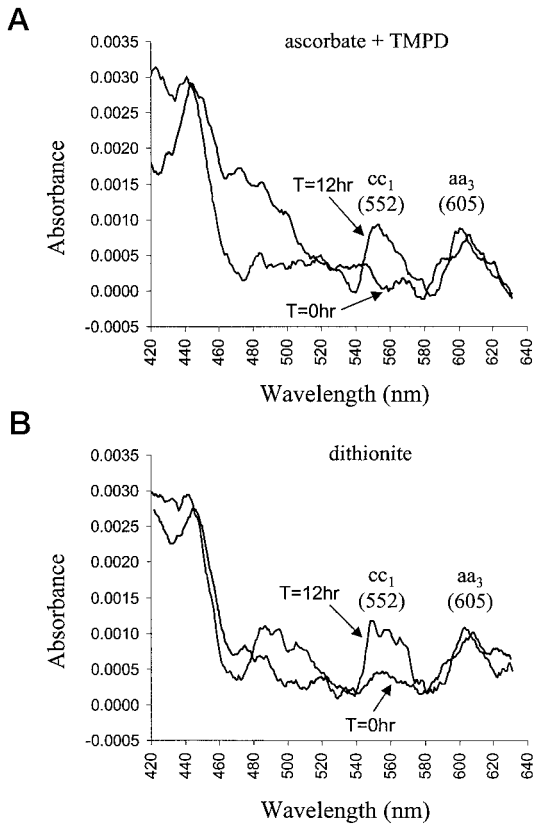

FIG. 3. **Scanning spectrophotometric analysis of cytochrome heme content.** *A,* difference spectra were obtained on whole cell extracts from BALB/3T3 cells serum-stimulated for 0 and 12 h using ascorbate + TMPD as reducing agents. *B,* difference spectra obtained with the same extracts as used above, except using dithionite a general reducing agent. The heme $c + c_1$ and $a + a_3$ peaks at 552 and 605 nm, respectively, are indicated above each spectrum.

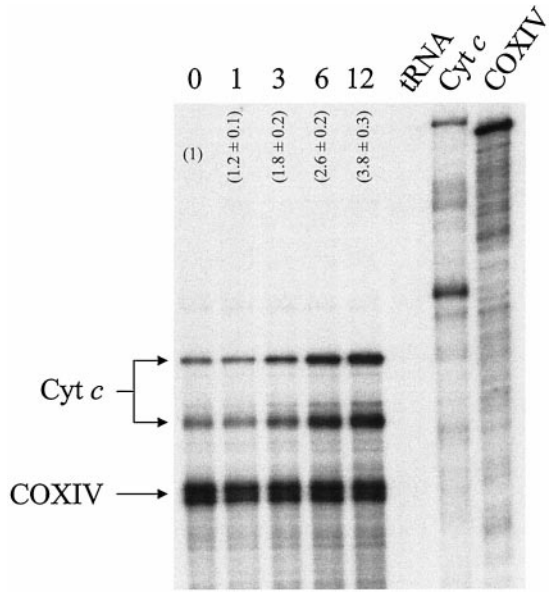

FIG. 4. **Comparison of cytochrome *c* and COXIV mRNA levels through a time course of serum stimulation.** RNase protection assay was performed on BALB/3T3 total RNA obtained from cells that were serum-stimulated for the indicated times. *Arrows* mark the positions of cytochrome *c* and COXIV RNase protection products. Lanes containing undigested probes and tRNA negative control are also indicated. *Numbers* in parentheses denote the fold induction of cytochrome *c* mRNA normalized to that of COXIV mRNA ± the standard error of the mean for three independent experiments.

TABLE II
Serum stimulation of cytochrome c promoter constructs

Construct[a]	Promoter activity[b]		
	+ serum	− serum	+/−[c]
pGL3RC4/−326	100	24.5 ± 4.1	4.1 ± 0.7
pGL3RC4/−326; CREDMI	28.8 ± 6.4	13.4 ± 2.2	2.2 ± 0.1
pGL3RC4/−326; NRF−1Mut	45.4 ± 10.8	19.5 ± 5.2	2.3 ± 0.1
pGL3RC4/−326; CREDM1/ NRF−1Mut	2.9 ± 0.3	2.1 ± 0.1	1.4 ± 0.1
pGL3RC4/−66	1.2 ± 0.4	0.9 ± 0.1	1.3 ± 0.2

[a] Constructs were derived from the rat cytochrome *c* promoter as described under "Experimental Procedures."

[b] Activities are expressed as a percentage of the activity derived from the wild-type cytochrome *c* promoter in pGL3RC4/−326. Values are the average of six separate determinations ± standard error and are normalized for transfection efficiency as described under "Experimental Procedures."

[c] Ratio of promoter activity produced by each construct upon serum stimulation (+ serum) and serum deprivation (− serum).

acting elements (pGL3RC4/-326) (Table II). This induction was eliminated by a large deletion (pGL3RC4/-66) removing the major regulatory elements in the 5′-flanking DNA.

CREs have been found to contribute to the induction of serum-responsive genes (39, 40). Two such elements within the cytochrome *c* promoter bind the transcription factor CREB and account for cAMP induction of cytochrome *c* mRNA (19). In addition, the promoter has a recognition site for NRF-1, an activator of nuclear genes involved in respiratory chain expression (18, 21). Point mutations in these elements were tested for their effects on serum induction of promoter activity. Elimination of both CREs (pGL3RC4/-326;CREDM1) reduced the serum inducibility to about half of the level observed with the full promoter (pGL3RC4/-326) (Table II). Likewise, mutation of the NRF-1 site alone (pGL3RC4/-326;NRF-1Mut) also reduced the serum response by half. Moreover, a construct with point mutations in all three elements (pGL3RC4/-326;CREDM1/NRF-1Mut) was almost completely unresponsive to serum. The Sp1 sites within the first intron (18) did not contribute to serum stimulation because they are present in the triple mutant (pGL3RC4/-326;CREDM1/NRF-1Mut) and in the −66 deletion (pGL3RC4/-66), which were both unresponsive. In addition, these sites were also mutated in the context of the full promoter and found to have no effect on the response to serum (not shown). The results establish that both CREB and NRF-1 recognition sites contribute equally to the serum induction of the cytochrome *c* promoter.

CREB and NRF-1 Activation through Sequential, Serum-induced Phosphorylation Mediate Cytochrome c Gene Expression—One means by which NRF-1 and CREB can mediate the serum induction of cytochrome *c* is through their activation by serum-dependent phosphorylation. Serum is known to activate CREB by bringing about its phosphorylation at serine 133 (41). Anti-CREB and anti-phospho-CREB antibodies were therefore used to measure CREB steady-state levels and the degree of CREB phosphorylation within the time frame of cytochrome *c* induction. The results show a rapid induction of CREB phosphorylation within 1 h of serum addition (Fig. 5). This induction was transient, in that it was significantly diminished by 6 h, and was not accompanied by any increase in the steady-state levels of CREB protein. The peak of CREB phosphorylation between 0 and 1 h immediately preceded the initial induction of cytochrome *c* mRNA between 1 and 3 h of serum addition (Fig. 4). These results are consistent with the contribution of CREs to the serum induction of the cytochrome *c* promoter.

We had previously shown that NRF-1 is phosphorylated on serine residues within a concise amino-terminal domain and that phosphorylation of recombinant NRF-1 *in vitro* increased

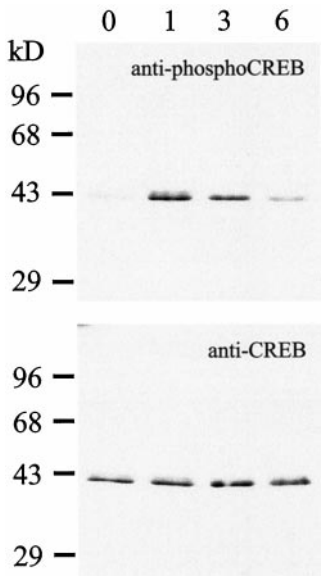

FIG. 5. **Analysis of CREB phosphorylation relative to CREB protein levels over a time course of serum stimulation.** An immunoblot using anti-phospho-CREB serum was performed on nuclear extracts obtained from BALB/3T3 cells that had been serum-stimulated for the indicated times (*upper panel*). An identical immunoblot prepared with anti-CREB serum as the primary antibody is also shown (*lower panel*). The molecular masses of protein standards are indicated at the *left*.

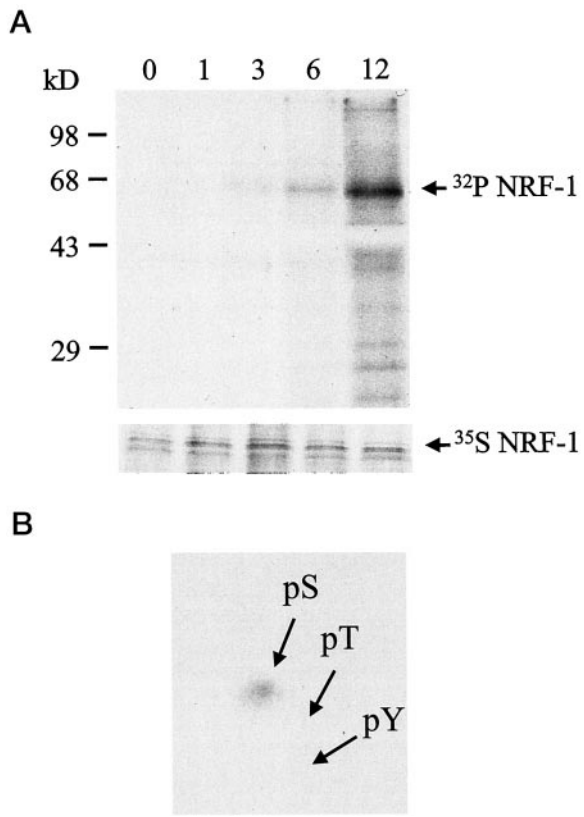

FIG. 6. **Analysis of NRF-1 phosphorylation over a time course of serum stimulation.** *A, in vivo* ^{32}P-labeled NRF-1 was immunoprecipitated from BALB/3T3 cells that had been serum-stimulated for the indicated times and electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis gel. Molecular mass standards are shown on the *left*. The *lower panel* shows *in vivo* ^{35}S-labeled NRF-1 that was immunoprecipitated from BALB/3T3 cells over the same time course of serum stimulation and electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis gel. *B,* phosphoamino acid analysis of *in vivo* ^{32}P-labeled NRF-1 immunoprecipitated from BALB/3T3 cells that had been serum-stimulated for 12 h. The *arrows* indicate the positions of phospho-serine (*pS*), phospho-tyrosine (*pY*), and phospho-threonine (*pT*) included as standards in the two-dimensional electrophoretic separation of the ^{32}P-labeled NRF-1 hydrolysate.

its DNA binding activity (24). It was therefore of interest to determine whether NRF-1 is induced or phosphorylated in response to serum stimulation. Cells were labeled with either ^{32}P or ^{35}S, and NRF-1 was immunoprecipitated from nuclear extracts over a time course of serum stimulation. The results show a pronounced increase in ^{32}P-NRF-1 beginning at about 3 h and extending through at least 12 h (Fig. 6*A*). No significant increase in ^{35}S-NRF-1 was detected in the same time interval, demonstrating that the steady-state levels of NRF-1 were unchanged. This was also confirmed by Western blotting (not shown). Phosphoamino acid analysis was performed on the induced ^{32}P-NRF-1 that had been excised and eluted from denaturing SDS gels. Only phosphoserine was detected in the hydrolyzed sample (Fig. 6*B*). Thus, the serine phosphorylation of NRF-1 was markedly induced within the time frame of cytochrome *c* induction and the increased respiratory activity. Unlike the transient phosphorylation observed with CREB, NRF-1 phosphorylation increased steadily between 3 and 12 h with the highest rate of increase occurring between 6 and 12 h.

Because of the endogenous levels of NRF-1 in mammalian cells, attempts to demonstrate that the *in vivo* phosphorylation of NRF-1 enhances its ability to *trans*-activate its target genes were unsuccessful (24). Here, this question was addressed using *Drosophila* SL2 cells, which have no detectable endogenous NRF-1. As shown in Fig. 7*A*, NRF-1 was abundantly phosphorylated in these cells. NRF-1/8xA is a derivative of NRF-1 in which the serine residues between amino acids 36 and 61 were mutated to alanines (24). In mammalian cells, identical phosphopeptides were observed for both endogenous and transfected NRF-1, and the 8xA mutation completely eliminated phosphorylation of the protein. Thus, the 8xA mutation defines a concise domain for serine phosphorylation *in vivo* (24). As observed in mammalian cells, NRF-1/8xA was not phosphorylated in *Drosophila* SL2 cells (Fig. 7*A*). Both wild-type and mutated proteins were equally expressed in transfected SL2 cells (Fig. 7*B*) and thus the difference in phosphorylation between the two derives from the availability of serine phosphorylation sites.

If NRF-1 phosphorylation contributes to enhanced target gene expression, the elimination of the serine phosphorylation sites utilized *in vivo* should reduce its ability to *trans*-activate. As shown in Fig. 8*A*, the cytochrome *c* promoter (RC4/-326) displayed a significant reduction in its *trans*-activation by the unphosphorylated mutant NRF-1 (NRF-1/8xA). Because this promoter is only partially NRF-1-dependent, the same experiment was performed using a truncated cytochrome *c* promoter whose expression is almost completely dependent on four tandem NRF-1 sites (4XNRF-1). In this case, mutation of the serine phosphorylation sites markedly reduced *trans*-activation of the promoter by NRF-1. The differences in activity did not result from different steady-state levels of expression because phospho and dephospho forms of NRF-1 were expressed at nearly identical levels in transfected cells (Fig. 8*B*). The results establish that phosphorylation of NRF-1 on the same serine residues that are normally phosphorylated *in vivo* upregulates the ability of NRF-1 to *trans*-activate its target genes. This is consistent with a requirement for an NRF-1 site in maximizing the serum induction of the cytochrome *c* promoter.

DISCUSSION

Many nuclear genes encoding the molecular constituents that are required for the synthesis and function of the mammalian respiratory apparatus have been characterized in re-

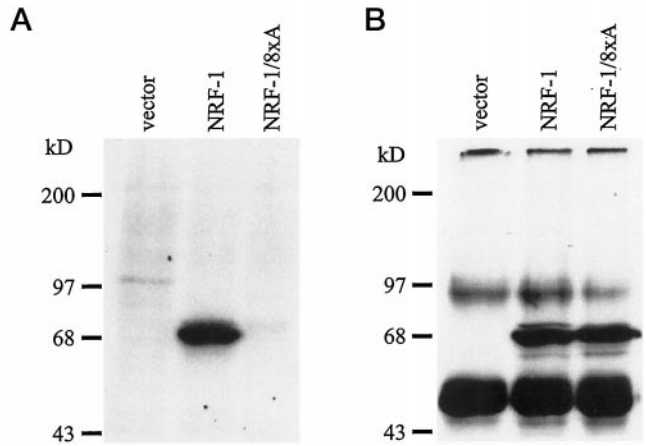

FIG. 7. ***In vivo* phosphorylation of NRF-1 expressed in SL2 cells.** *A,* SL2 cells were calcium phosphate transfected with the pPac5c (vector), pPac5cNRF-1, or pPac5cNRF-1/8xA. Transfected cells were *in vivo* labeled with [^{32}P]orthophosphate and the expressed NRF-1 or NRF-1/8xA was immunoprecipitated from labeled cell lysates with anti-NRF-1 serum. *B,* the same membrane in *A* was subjected to chemiluminescent detection of bound proteins using anti-NRF-1 serum as the primary antibody.

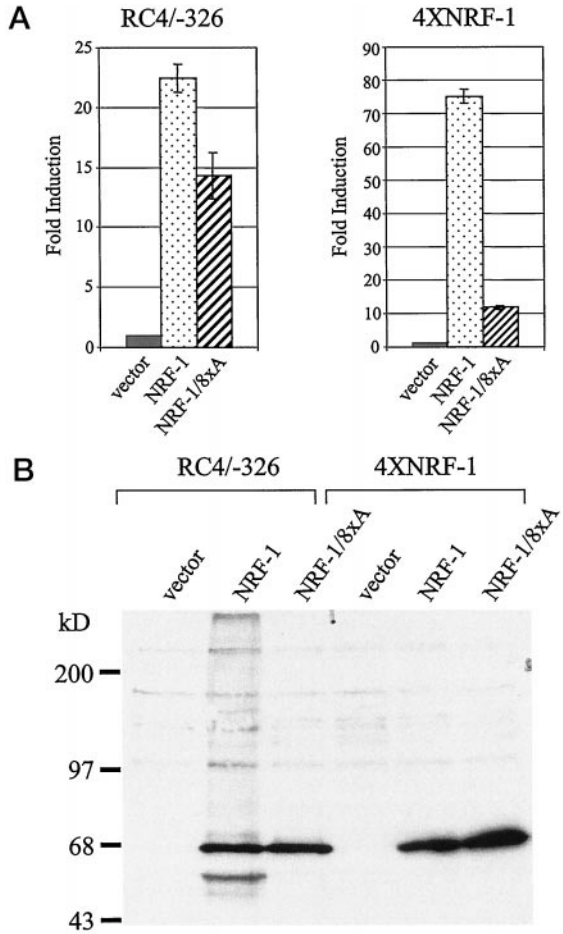

FIG. 8. **Effect of NRF-1 phosphorylation on its ability to transactivate NRF-1-responsive promoters in SL2 cells.** *A,* either pPac5c (vector) or pPac5c derivatives expressing NRF-1 or NRF-1/8xA were cotransfected into SL2 cells along with the NRF-1-responsive reporters RC4/-326 (*left panel*) or 4XNRF-1 (*right panel*). Results are represented as fold induction of the NRF-1 *trans*-activated reporter compared with that of empty vector. For results on RC4/-326, the two sample *t* test results were $p = 0.003$, and the standard error was determined with an $n \geq 5$. For results on 4XNRF-1, standard error was determined with an $n \geq 7$. *B,* an immunoblot was performed on total cell extracts from transfected cells used in *A* using anti-NRF-1 serum as the primary antibody. The molecular masses of protein standards are indicated at the *left*.

cent years. These include genes for the majority of respiratory chain subunits as well as for essential regulatory factors governing the transcription and replication of the mitochondrial genome (11–13). In addition, a number of nuclear transcription factors that participate in the expression of many of these genes have been identified. In particular, NRF-1 and NRF-2 act on overlapping subsets that include the majority of known nuclear genes involved in the biogenesis of the respiratory chain (13). However, not all such genes are governed by common transcription factors, and there is little evidence for coordinate transcriptional expression of a large number of functionally related genes. This raises the possibility that gene-specific regulatory mechanisms that depend on promoter context are important in controlling the expression of the respiratory apparatus.

Although the mechanisms regulating respiratory gene expression in response to oxygen and nonfermentable substrates are well characterized in yeast (15), little is known of the signaling pathways that mediate the expression of the respiratory chain in mammalian cells. The cAMP induction of the cytochrome *c* promoter through CREB, a cAMP-responsive transcription factor, may signal the availability of metabolic fuels to the respiratory chain (13, 19). Peptide hormones utilize cAMP-dependent pathways in the release of glucose and triacyglycerols, which are rich sources of oxidative energy (42). The presence of CREB recognition sites in the cytochrome *c* promoter makes it unique among the promoters of nuclear genes specifying the respiratory chain. The CREB sites help mediate a rapid response to both serum and cAMP in keeping with a unique role for cytochrome *c* in regulating cellular respiration. Differential induction of cytochrome *c* mRNA relative to that of COXIV is also mediated through thyroid hormone in rat liver (32). Although this induction is unlikely to result from a direct activation of the promoter by thyroid hormone receptor, it may reflect a need to up-regulate cytochrome *c* relative to other respiratory subunits. In addition to regulating energy production, the differential control of cytochrome *c* may affect the levels of reactive oxygen species. Cytochrome *c* has been implicated in the oxidation of superoxide anion to molecular oxygen. This property, along with its ability to induce apoptosis, may reflect a general antioxidant function for cytochrome *c* (17).

It has been argued that cellular energy production is regulated through cytochrome oxidase, the terminal step in electron transport (16). One means of implementing a long term change in respiratory capacity is through the differential regulation of cytochrome *c*, a dissociable electron carrier that is a key participant in the cytochrome oxidase reaction. Such a mechanism would allow cells to regulate their respiratory capacity by controlling a single component rather than by synthesizing, localizing, and assembling the integral membrane respiratory complexes. Recent threshold studies using both primary and established human cell lines indicate that cytochrome oxidase is in low excess for glutamate/malate-dependent respiration and that cytochrome *c* may not be in excess over the level required for cytochrome oxidase activity (43). This is consistent with the results presented here whereby cellular respiration is increased through the differential transcriptional induction of cytochrome *c*.

It should be noted that mRNAs for COXI, ATP synthase β, and adenine nucleotide transporter 2 have been reported to be induced by serum. However, the effects of these mRNA inductions on the activities or amounts of the complexes they encode were not determined (44). Our results indicate that there are no significant changes in the level or activity of cytochrome oxidase within 12 h of serum stimulation. However, it is possible

that the induction or modification of key subunits in other respiratory complexes may help mediate the respiratory response to serum. For example, the 18-kDa subunit of complex I is phosphorylated by protein kinase A (45), and this phosphorylation may regulate the activity of complex I (51). A similar activation of complex I may contribute to the increased respiration we observe here between 0 and 3 h of serum stimulation, which precedes the major increase in cytochrome *c*-dependent respiration.

We establish that the marked increase in cellular respiration in response to serum stimulation of quiescent cells is associated with the sequential activation of CREB and NRF-1. CREs are known to mediate a serum response that is independent of cAMP signaling (39, 40). Moreover, serum growth factors, including nerve growth factor, fibroblast growth factor, epidermal growth factor, and insulin, can signal through RAS and the family of mitogen activated protein kinases (46–49). Stimulation of cells by each of these mitogens results in the phosphorylation of CREB at serine 133. Although CREB phosphorylation can account for cytochrome *c* promoter induction through the CREs, it is possible that other members of the activating transcription factor/CREB family are involved.

Although both CREB and NRF-1 are activated through serine phosphorylation, they are not functionally equivalent. The phosphorylation of CREB is both rapid and transient indicating that it contributes to the early induction of cytochrome *c* within 3 h of serum addition. By contrast, although NRF-1 phosphorylation is evident within 3 h, the peak occurs between 6 and 12 h of serum addition and is maintained for at least 24 h (not shown), long after the onset of DNA replication. These results indicate that NRF-1 functions both in inducing gene expression and in maintaining cytochrome *c* levels in actively dividing cells. Activated NRF-1 may also contribute to the synthesis of other respiratory chain constituents later in the cell cycle. Thus, the phosphorylation of NRF-1 may be an important regulatory event in the transition from quiescence to active cell division.

Serine phosphorylation by casein kinase II (CKII) within a concise amino-terminal domain in NRF-1 has been shown to increase its DNA binding activity *in vitro* (24). However, the increased expression of reporter genes mediated by phospho-NRF-1 in SL2 cells represents the first demonstration that phosphorylation of NRF-1 enhances its ability to *trans*-activate gene expression. CKII phosphorylates the same NRF-1 serine residues *in vitro* as those phosphorylated *in vivo* (24). Interestingly, CKII activity undergoes oscillations when quiescent fibroblasts are serum-stimulated (50). After an early spike, a major increase in CKII activity occurs between 4 and 12 h after serum addition coinciding with the major increase in NRF-1 phosphorylation observed here. These results suggest that CKII or a related cell cycle-regulated kinase may be responsible for the serum-dependent activation of NRF-1.

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Sequential Serum-dependent Activation of CREB and NRF-1 Leads to Enhanced Mitochondrial Respiration through the Induction of Cytochrome *c*

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