c-MYC apoptotic function is mediated by NRF-1 target genes

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A detailed understanding of the signaling pathways by which c-Myc elicits apoptosis has proven elusive. In the current study, we have evaluated whether the activation of the mitochondrial apoptotic signaling pathway is linked to c-Myc induction of a subset of genes involved in mitochondrial biogenesis. Cytochrome c and other nuclear-encoded mitochondrial genes are regulated by the transcription factor nuclear respiratory factor-1 (NRF-1). The consensus binding sequence (T/C)GCGCA(C/T)GCGC(A/G) of NRF-1 includes a noncanonical CA(C/T)GCG Myc:MAX binding site. In this study, we establish a link between the induction of NRF-1 target genes and sensitization to apoptosis on serum depletion. We demonstrate, by using Northern analysis, transactivation assays, and in vitro and in vivo promoter binding assays that cytochrome c is a direct target of c-Myc. Like c-Myc, NRF-1 overexpression sensitizes cells to apoptosis on serum depletion. We also demonstrate that selective interference with c-Myc induction of NRF-1 target genes by using a dominant-negative NRF-1 prevented c-Myc-induced apoptosis, without affecting c-Myc-dependent proliferation. These results suggest that *c-myc* expression leads to mitochondrial dysfunction and apoptosis by deregulating genes involved in mitochondrial function.

[*Keywords*: Apoptosis; c-MYC; NRF-1; mitochondrial biogenesis]

Supplemental material is available at http://www.genesdev.org.

Received August 13, 2002; revised version accepted November 21, 2002.

The c-Myc oncogene, which is dysregulated in many cancers (for review, see Nesbit et al. 1999), functions as a positive regulator of cell proliferation and growth (for review, see Dang 1999; Grandori et al. 2000; Nasi et al. 2001). One of the paradoxes of *c-myc* overexpression is the resultant sensitization of cells to apoptosis under conditions of serum deprivation or after treatment with DNA-damaging agents. (Evan et al. 1992). Understanding the molecular mechanism underlying c-Myc-induced apoptosis may thus have important implications for treatment of Myc-related cancers (for review, see Prendergast 1999). Earlier studies have demonstrated that c-Myc-induced apoptosis involves loss of mitochondrial membrane potential (Fulda et al. 1999), the release of cytochrome c (Juin et al. 2000) and AIF (Daugas et al. 2000), and activation of caspase-3 (Kagaya et al. 1997). These events are associated with mitochondrial dysfunction, and this apoptosis is inhibited by the Bcl-2 oncoprotein, (Bissonnette et al. 1992). Inhibition of c-Myc apoptosis by Bcl-2 may be linked to inhibition of cytochrome c release, and regulation of mitochondrial membrane potential and permeability (Kluck et al. 1997). Although events downstream of the site of action of Bcl-2 are fairly well characterized, the mechanism by which *c-myc* overexpression disrupts normal mitochondrial function, leading to the release of cytochrome c, remains unexplained.

Production of functional mitochondria requires proteins derived from both nuclear and mitochondrial genomes (for review, see Poyton and McEwen 1996) and is tightly regulated by a series of transcription factors (for review, see Scarpulla 2002). The DNA recognition site for one of these factors includes a core domain CA(T/C)GCG, which is identical to a noncanonical Myc:MAX binding site (Virbasius et al. 1993; Grandori and Eisenman 1997). This factor, called nuclear respiratory factor 1 (NRF-1), is a member of a unique family of evolutionarily conserved transcription factors that play critical roles in eukaryotic development. These include the zebrafish *nrf* (Becjker et al. 1998) and the *Drosophila* erect wing gene product (*ewg*; DeSimone and White 1993), which are both essential genes, as homozygous knockouts are embryonic lethals (DeSimone and White 1993; Becjker et al. 1998). Human NRF-1 has been shown to transactivate the promoters of a number of mitochondrial-related genes (for review, see Scarpulla 2002). NRF-1 binds as a homodimer to the palindromic GC-rich sequence (T/C)GCGCA(C/T)GCGC(A/G), stable high-affinity binding requires a tandem direct repeat of the T/CGCGCA motif, and maximal binding activity re-

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1032503.

quires phosphorylation (Virbasius et al. 1993; Gugneja and Scarpulla 1997).

One of the most well-studied NRF-1 target genes is cytochrome c, a gene shown to play a critical role in both electron transport and apoptosis. Cytochrome c-null mice are not viable beyond day 10, owing to respiratory insufficiency, and cells derived from these embryos are resistant to apoptosis after a variety of stimuli (Li et al. 2000). The induction of cytochrome c on the addition of serum is associated with enhanced respiration (Herzig et al. 2000). Similarly, other genes are coregulated in response to changes in growth state (for review, see Nelson et al. 1995), leading to changes in the proliferation or differentiation of mitochondria. NRF-1, in combination with other transcription factors, controls the coordinated expression of genes essential for mitochondrial function. This ensures that the cell maintains the correct stoichiometry of respiratory complex subunits and a means for their import and correct assembly in the mitochondria (for review, see Poyton and McEwen 1996). A disruption of this critical balance has a detrimental effect, and examples exist of mitochondrial dysfunctions that result from both reduced (for review, see Schapira and Cock 1999) and increased expression of genes involved in mitochondrial function, such as adenine nucleotide translocase-1 (Bauer et al. 1999) and the mitochondrial hinge protein (Okazaki et al. 1998).

The possibility that c-Myc could stimulate NRF-1 target genes, as a result of similarity in binding sites, has prompted the current work. In this study, we sought to determine if the mitochondrial events that underlie c-Myc-induced apoptosis are initiated by the inappropriate expression of NRF-1 target genes. Here we demonstrate that c-Myc is capable of selectively up-regulating NRF-1 target genes. We also show that under conditions shown to trigger c-Myc-induced apoptosis, overexpression of NRF-1 also sensitizes cells to apoptosis. Finally, we demonstrate that a dominant-negative NRF-1 selectively inhibits apoptosis induced by c-Myc while maintaining the proliferative effects of c-Myc. These results are discussed in the context of broader questions regarding the biology of cell death and the role played by c-Myc in cellular metabolism.

Results

Induction of NRF-1 target genes in Myc-ER^{TM} cell lines

In the current study, we have used the conditional Myc-ER^{TM} protein (Littlewood et al. 1995) in an NIH3T3 cell line to evaluate the ability of c-Myc to induce NRF-1 target genes. This protein is a chimeric fusion protein of full-length c-Myc and a mutant estrogen receptor (ER^{TM}). This receptor has a deletion allowing activation by 4-hydroxytamoxifen (4-OHT) but not by endogenous estrogens. We evaluated the induction of NRF-1 target genes over an 8-h time course in the presence of cycloheximide and 4-OHT, as previous studies have demonstrated that maximal transactivation by c-Myc occurs within this timeframe (Wu et al. 1999). Prior to induction, cells were serum depleted for 48 h. We chose to look at two NRF-1-regulated genes, *cytochrome c* and *mtTFA*, as potential c-Myc targets. The NRF-1 binding site in the cytochrome c promoter contains a noncanonical Myc:MAX binding sequence (CATGCG), whereas no known c-Myc binding site is present in the mtTFA promoter. We also tested the induction of *NRF-1* to determine if c-Myc influenced the expression of this transcription factor. These experiments were done in the presence of cycloheximide to allow an analysis of direct targets of c-Myc. Cytochrome c levels in the Myc-ER^{TM} line showed a significant induction at 8 h after 4-OHT addition, whereas no induction was seen in the vector control line (Fig. 1A,B). In contrast, mtTFA and NRF-1 mRNA levels were not significantly induced on 4-OHT addition (Fig. 1A), indicating that c-Myc does not transactivate these transcription factors. These results provide further evidence that increases in cytochrome c transcripts are a direct result of c-Myc induction. Results of protein labeling studies, performed under serum-deprived conditions, demonstrate that c-Myc expression also results in increases in cytochrome c protein synthesis. Protein levels of cytochrome c were two- to threefold greater than those of control cells grown under similar conditions (Fig. 1G).

Induction of cytochrome c in a c-myc$^{-/-}$ *cell line*

To further test the role of c-Myc in the induction of cytochrome c, we evaluated the expression of this gene in *c-myc*$^{-/-}$ cells (Mateyak et al. 1997). In these studies, we compared the expression profile of *c-myc*$^{-/-}$ (KO) cells with those of the wild-type parental rat fibroblast cell line TGR1 *c-myc*$^{+/+}$ and the KO line reconstituted with c-myc (Komycer). Cells were serum-starved for 48 h, and a time course of induction of cytochrome c was determined after serum addition. NRF-1 is functionally down-regulated with serum deprivation, and serum induction of cytochrome c promoter-reporter constructs requires sequential activation of CREB and NRF-1 (Herzig et al. 2000). As illustrated (Fig. 1C), there is a sustained and increased induction of cytochrome c over the 8-h time course in the *c-myc*$^{+/+}$ cell line. In contrast, in the *c-myc*$^{-/-}$ line, the initial induction of cytochrome c seen at 2 h is not sustained. These results suggest that c-Myc may cooperate with CREB and NRF-1 in the regulation of cytochrome c induction on serum addition.

Myc:MAX heterodimers bind to the cytochrome c promoter

Electrophoresis mobility shift assays (EMSAs) were used to determine if Myc:MAX heterodimers could bind to the NRF-1 site in the cytochrome c promoter. Representative results (Fig. 1D) show that ^{32}P-labeled dsDNA containing the cytochrome c NRF-1 binding site was specifically bound by Myc:MAX heterodimers (Fig. 1D, lane 4). Myc:MAX binding to the wild-type NRF-1 site was effectively competed with an excess of unlabeled

canonical c-Myc binding sequence (E box CACGTG; Fig. 1D, lane 6) or unlabeled wild-type cytochrome c dsDNA (Fig. 1D, lane 11). Binding was not inhibited in the presence of a mutated E box (Fig. 1D, lanes 7,8) or by cytochrome c dsDNA with a mutated NRF-1 binding site (Fig. 1D, lanes 12–14).

Myc binds to cytochrome c in vivo

To further substantiate our observations of Myc-dependent serum stimulation of cytochrome c expression, we performed chromatin immunoprecipitation (ChIP) assays with TGR and KO cells that were serum stimulated for 4 h. We chose 4 h, as the association of Myc with genomic target sites has been shown to reach maximal levels at this time point (Frank et al. 2001; Zeller et al. 2001). Albumin was used as a negative control for binding. The 348-bp region of the cytochrome c promoter amplified by PCR contains the single CATGCG motif of the NRF-1 binding site and no other known Myc:MAX binding motifs. Results from these studies demonstrate that Myc binds to cytochrome c in vivo on serum addition, leading to a 12-fold increase in Myc binding after 4 h. Similar analysis for a second NRF-1 target gene, COX5b, also showed an increase in Myc binding (Fig. 1E).

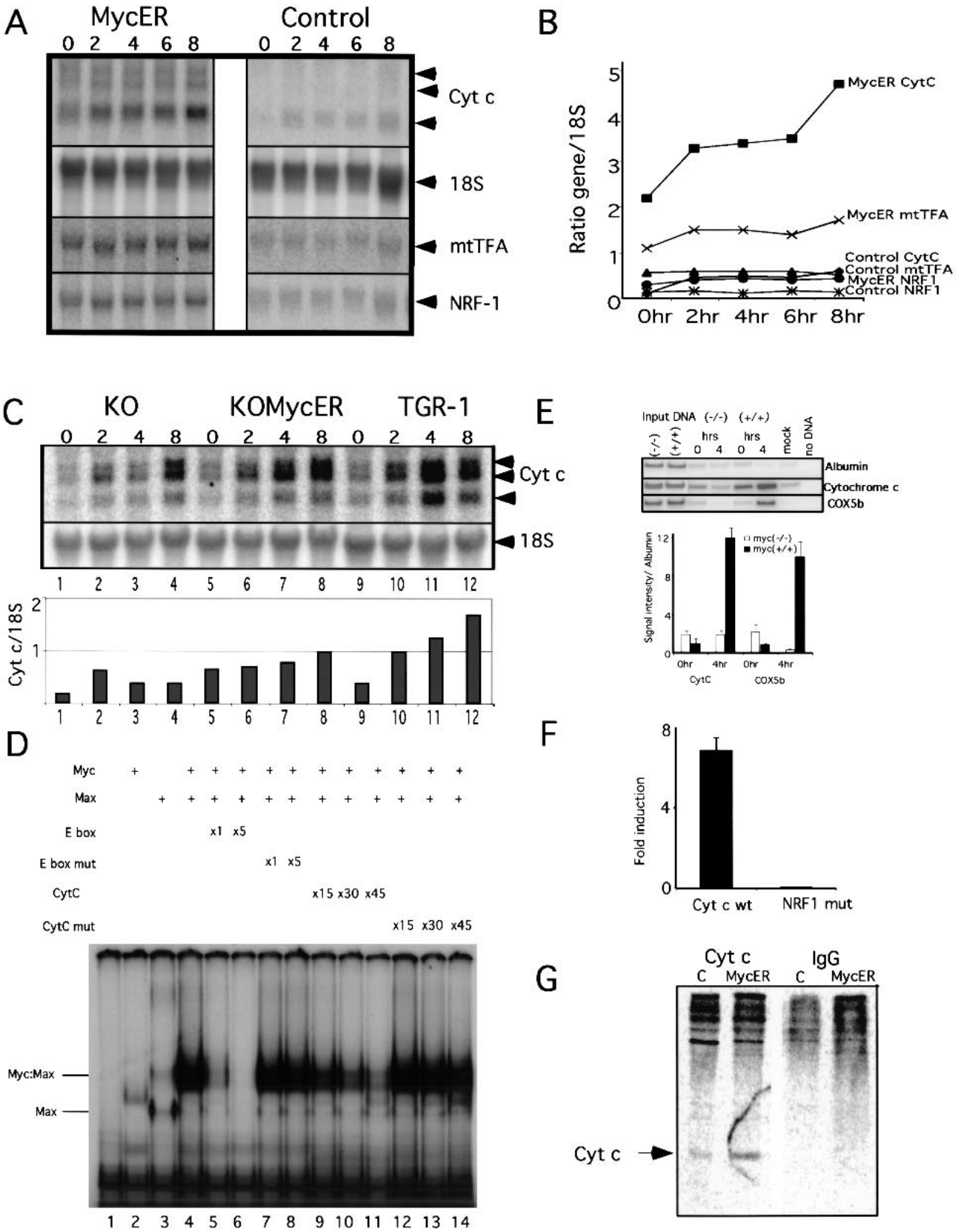
c-Myc transactivates the cytochrome c *promoter through the NRF-1 binding site*

The above results from Northern, ChIP, and EMSA analysis suggested that cytochrome c may be directly transactivated by the binding of c-Myc to the NRF-1 binding site. To further evaluate the importance of the NRF-1 binding site in c-Myc transactivation of cytochrome c, we used reporter assays. The cytochrome c luciferase reporter constructs used here contain either a wild-type (pGL3RC4/-326) or mutant NRF-1 binding site (pGL3RC4/-326 NRF-1 mut; Evans and Scarpulla 1989; Xia et al. 1997). We first tested the effects of increasing concentrations of c-Myc on transactivation of the wild-type promoter. Results of these experiments demonstrate that c-Myc transactivates cytochrome c in a concentration-dependent fashion (data not shown). Both pGL3RC4/-326 and pGL3RC4/-326 NRF-1 were then tested for transactivation by using c-Myc, and results of this assay clearly indicate that mutation of the NRF-1 binding site inhibited the ability of c-Myc to transactivate cytochrome c (Fig. 1F). These results demonstrate that both c-Myc and NRF-1 share the ability to transactivate cytochrome c.

Overexpression of NRF-1 induces apoptosis after serum depletion

The above results, demonstrating that c-Myc transactivates cytochrome c suggests that other NRF-1 target genes may also be regulated by c-Myc. To test if the induction of NRF-regulated genes sensitized cells to apoptosis, we examined the effects of overexpression of *NRF-1* in serum-deprived NIH3T3 cells. The estrogen receptor fusion system was again used in these experiments, and NIH3T3 cells were transduced with a retroviral vector containing NRF-1-ER^{TM}. Several independent clones were tested and shown to express the NRF-1-ER^{TM} fusion protein (Fig. 2A), and Northern analysis demonstrates an increase in the expression of the NRF-1 target genes *cytochrome c* and *mtTFA* (Fig. 2B). These clones showed high levels of expression of these genes, even in the absence of 4-OHT, and are thus leaky. Under serum-deprived conditions, the expression of NRF-1 also increased the level of cytochrome c protein compared with the control (Fig. 2C). Detailed analysis of three independent clones demonstrate that the overexpression of NRF-1 triggered apoptosis on serum depletion as measured by sub-G1 populations in propidium iodide flow cytometry assays (PI FACS; Fig. 2D). Electron microscopy revealed enlarged mitochondria in the serum-deprived NRF-1 cell lines with extensive rupture of mitochondria (Fig. 2E). These mitochondria had a dense matrix with indistinct cristae, which was also characteristic of mitochondria in cells undergoing c-Myc-induced apoptosis (Fig. 6B, below).

Figure 1. c-Myc transactivation of the NRF-1 target gene cytochrome c. (*A*) Northern analysis of Myc-ER^{TM} and vector control samples isolated at 0, 2, 4, 6, and 8 h after 4-OHT treatment in the presence of 10 μg/mL cycloheximide. Cells had been serum depleted for 48 h prior to cycloheximide and 4-OHT addition. There are three cytochrome c transcripts of 1.4, 1.1, and 0.7 kb. (*B*) Graph of the relative level of induction of mRNA over time in Myc-ER^{TM} and vector control lines. Data is corrected for loading by using 18S. This data is representative of three separate experiments. (*C*) Induction of cytochrome c on serum addition in *c-myc*$^{-/-}$ and *c-myc*$^{+/+}$ cell lines after 48 h of serum depletion. This experiment was repeated twice with similar results. (*D*) EMSA assay of the ability of Myc:MAX and MAX:MAX to bind to the NRF-1 binding site in the cytochrome c promoter. Myc and MAX were both recombinant forms and used alone at a concentration of 25 ng Myc (lane *2*) and 7.5 ng MAX (lane *3*) or were combined (lane *4*). Samples *5–14* show competition with unlabeled dsDNA samples, including E box (lanes *5,6*) a mutated E box (lanes *7,8*), wild-type cytochrome c (lanes *9–11*), and mutant cytochrome c DNA (lanes *12–14*). (*E*) PCR of input chromatin for *c-myc*$^{-/-}$ and *c-myc*$^{+/+}$ and of chromatin immunoprecipitated with c-Myc antibody, nonimmune serum (mock), and no DNA by using primers for cytochrome c, COX5b, and the negative control albumin. The histograms denote the level of Myc association with cytochrome c and COX5b compared with the albumin control for three replicas. (*F*) Histograms representing the ability of c-Myc to regulate the cytochrome c promoter through the NRF-1 site or a mutant site in which CATGCG has been mutated to CATTAG. Transfections were done in triplicate, and results are expressed as the fold increase in luciferase activity compared with the empty vector control (pRcCMV) corrected for transfection efficiency, and are representative of four separate experiments. (*G*) Immunoprecipitation with cytochrome c demonstrates increases in the level of cytochrome c protein synthesis on the activation of c-MYC in serum-deprived cells. Control and MycER cell lysates were immunoprecipitated in the presence of mouse IgG conjugated to agarose or cytochrome c coupled to protein G-Sepharose.



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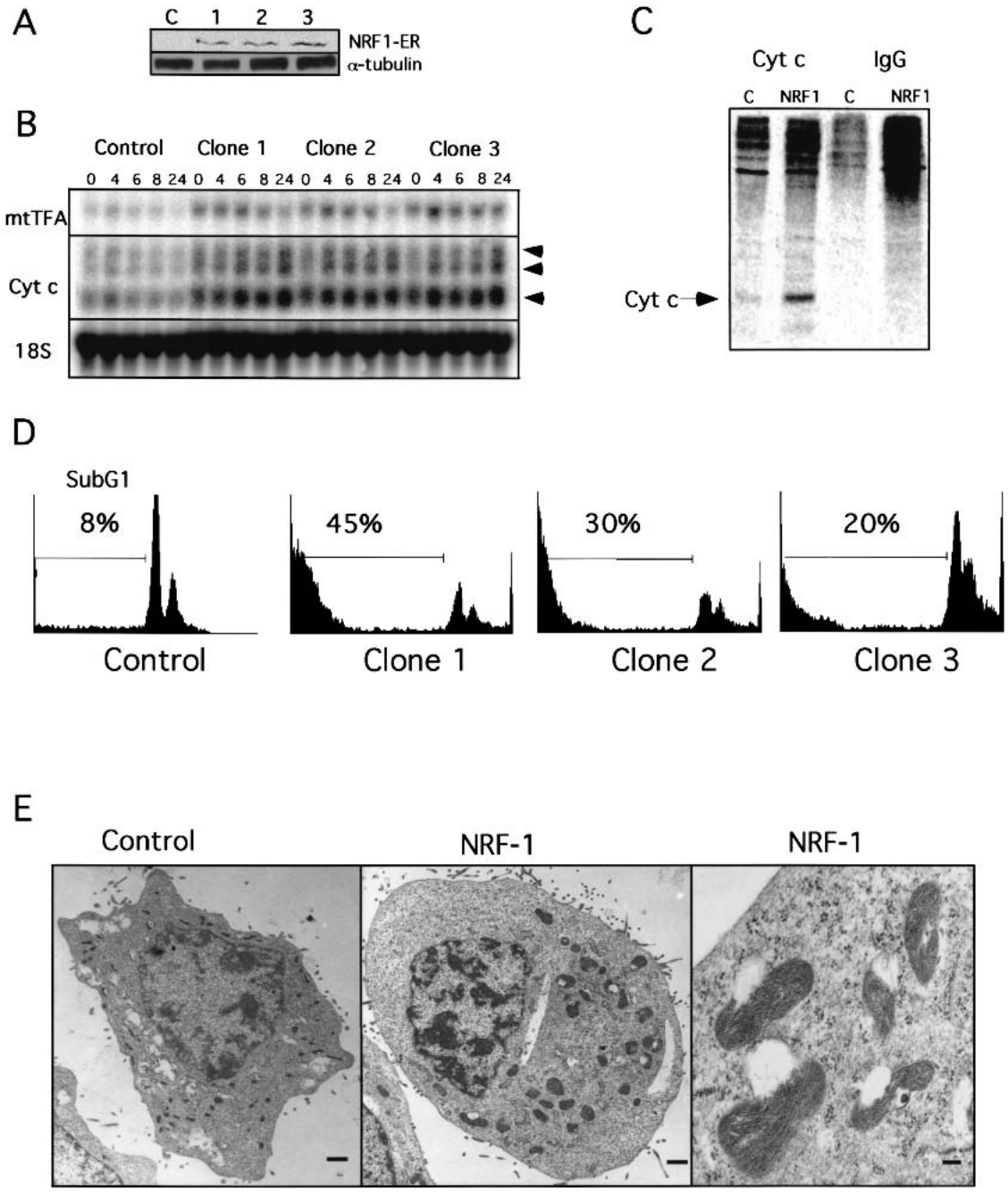


Figure 2. Overexpression of the transcription factor NRF-1 induces apoptosis in serum-depleted cells. (*A*) Western analysis for three clones expressing the NRF-1-ER fusion protein. (*B*) Northern analysis of clones overexpressing *NRF-1* over a time course of 24 h in the presence of 4-OHT and cycloheximide. (*C*) Immunoprecipitation with cytochrome c demonstrates increases in the level of cytochrome c protein synthesis in NRF-1-expressing cells under serum deprivation. Control and NRF-1 cell lysates were immunoprecipitated in the presence of mouse IgG conjugated to agarose or cytochrome c coupled to protein G-Sepharose. (*D*) PI FACS analysis indicating the percentage of sub-G1 cell populations found in three independently derived cell lines overexpressing *NRF-1* after 24 h in 0.5% serum. Approximately 10^5 cells were analyzed in triplicate for each sample, and these results are representative of three separate experiments. (*E*) Electron microscopy analysis of cells from control and NRF-1-overexpressing cells after 8 h in 0.5% serum. Bars: *left* and *middle* panels, 2 μm; *right* panel, 200 nm.

Bcl-2 expression prevents apoptosis induced by NRF-1

c-Myc-induced apoptosis is inhibited in the presence of Bcl-2 (Bissonnette et al. 1992). If c-Myc and NRF-1 share common apoptotic pathways, then Bcl-2 would also be predicted to inhibit NRF-1-induced apoptosis. To test this, the *Bcl-2* gene was introduced into an NRF-1-ER^{TM} cell line, and a series of clones were isolated and selected for analysis based on Bcl-2 expression (data not shown). A number of clones were evaluated for viability after

serum depletion. The presence of Bcl-2 resulted in a significant drop in the percentage of sub-G1 cells by FACS analysis (Fig. 3A), and EM micrographs clearly showed that the mitochondria in these cells were intact 24 h after initiation of serum depletion (Fig. 3C). These results are consistent with the hypothesis that both NRF-1 and c-Myc sensitize cells to apoptosis by inducing mitochondrial dysfunction.

A dominant-negative mutant of NRF-1 inhibits Myc-induced apoptosis

The above results suggest that both NRF-1 and c-Myc share target genes that are involved in sensitizing cells to apoptosis. To directly test this hypothesis, an ER fusion of the previously characterized dominant-negative mutant of NRF-1 (DNNRF-1; Wu et al. 1999) was transduced into a Myc-ER^{TM} cell line. As both c-Myc and the DNNRF-1 were ER fusions, the addition of 4-OHT would then allow simultaneous activation of both proteins. Clones recovered after selection in G418 were tested for the presence of DNNRF-1 and for apoptosis on serum depletion in the presence of 4-OHT. A series of independent clones were found to show high levels of DNNRF-1 (data not shown), and three were chosen for more detailed analysis.

Northern analysis was undertaken to compare expression of genes with known NRF-1 binding sites in the Myc-ER^{TM} and DNNRF-1-ER^{TM}/Myc-ER^{TM} cells, when grown in serum depleted medium in the presence of cycloheximide and 4-OHT. The expression of cyclin D2 and CAD, which both contain a canonical Myc:MAX binding site, was also evaluated. The activation of Myc-ER^{TM} resulted in increases in transcription of the NRF-1 target genes cytochrome c, COX5b, and COX6A1. The coactivation of DNNRF-1-ER^{TM} in Myc-ER^{TM} cells inhibited the induction of genes with NRF-1 binding sites but not the induction of cyclin D2 or CAD (data not

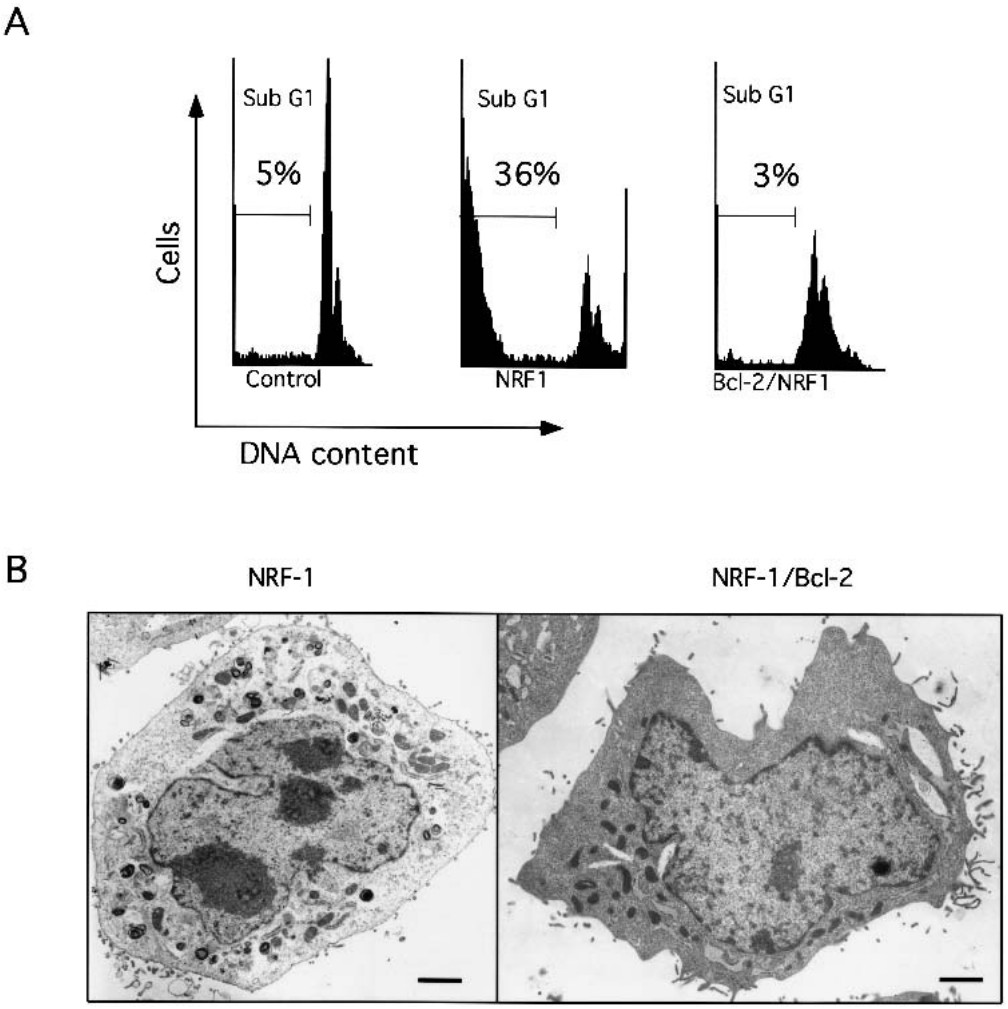


Figure 3. The antiapoptotic protein Bcl-2 protects cells from NRF1-induced apoptosis. (*A*) PI FACS analysis of vector control and *NRF-1*- and *Bcl-2/NRF-1*-expressing cell lines, indicating the percentage of sub-G1 cells in the population after 24 h in 0.5% serum. A minimum of 10^5 cells were analyzed in triplicate for each sample, and these results are representative of two separate experiments. (*B*) Electron microscopy analysis of NRF-1-ER^{TM} and Bcl-2/NRF-1-ER^{TM} cell lines after 24 h in 0.5% serum. Bars, 2 nM.

shown), which have canonical Myc:MAX binding site (Fig. 4A,B; Boyd and Farnaham 1997; Bouchard et al. 1999). These results demonstrate that the dominant-negative NRF-1 protein effectively suppressed c-Myc induction of the NRF-1 target genes, cytochrome c COX5b and COX6A1.

Further studies with these lines revealed that activation of DNNRF-1-ER^{TM} antagonized c-Myc-induced apoptosis on serum depletion. Results from flow cytometry analysis demonstrate that coactivation of DNNRF-1-ER^{TM} results in a reduction in the percentage of sub-G1 cells, from 45% to 5% (Fig. 5A), and Western analysis demonstrated an inhibition of caspase-3 cleavage (Fig. 5B). However, cell cycle progression was not affected, as the percentage of S-phase cells in DNNRF-1-ER^{TM}/Myc-ER^{TM} and Myc-ER^{TM} cell lines after 24 h of the 4-OHT addition were similar (Fig. 5C). As a further measure of the specific inhibition of c-Myc-induced apoptosis, the viability and proliferative ability of these lines was evaluated at 48 and 72 h after 4-OHT addition. At 72 h, only 13% of Myc-ER^{TM} cells were viable by trypan blue staining, whereas viability ranged from 64% to 90% for the DNNRF-1-ER^{TM}/Myc-ER^{TM} clones, compared with 94% for the vector control (Fig. 5D). Proliferation assays demonstrate two- to threefold increase in cell number for serum-deprived DNNRF-1-ER^{TM}/Myc-ER^{TM} cells with 4-OHT treatment compared with serum-deprived cells not treated with 4-OHT (Fig. 5E). These results provide further evidence that DNNRF-1-ER^{TM}/Myc-ER^{TM} cells remain viable and proliferate under serum-deprived conditions.

Phase contrast microscopy of cells after 48 h of 4-OHT treatment illustrates the morphological differences between these lines. The DNNRF-1-ER^{TM}/Myc-ER^{TM} lines grew as loosely adherent cells and were clearly different from both the control and the Myc-ER^{TM} cell line (Fig. 6A). These cells were shown to be viable by trypan blue staining (Fig. 5D). Electron microscopy studies revealed clear differences between Myc-ER^{TM} and DNNRF-1-ER^{TM}/Myc-ER^{TM} clones at the ultrastructural level (Fig. 6B). In contract to Myc-ER^{TM} cells, which showed obvious signs of apoptosis with intense cellular vacuolization and nuclear chromatin condensation, DNNRF-1-ER^{TM}/Myc-ER^{TM} cells showed none of these characteristics. In many of the Myc-ER^{TM} cells, the mitochondria had ruptured 48 h after 4-OHT addition. In cells with intact mitochondrial, these were normal in shape but showed no distinct cristae (Fig. 6B).

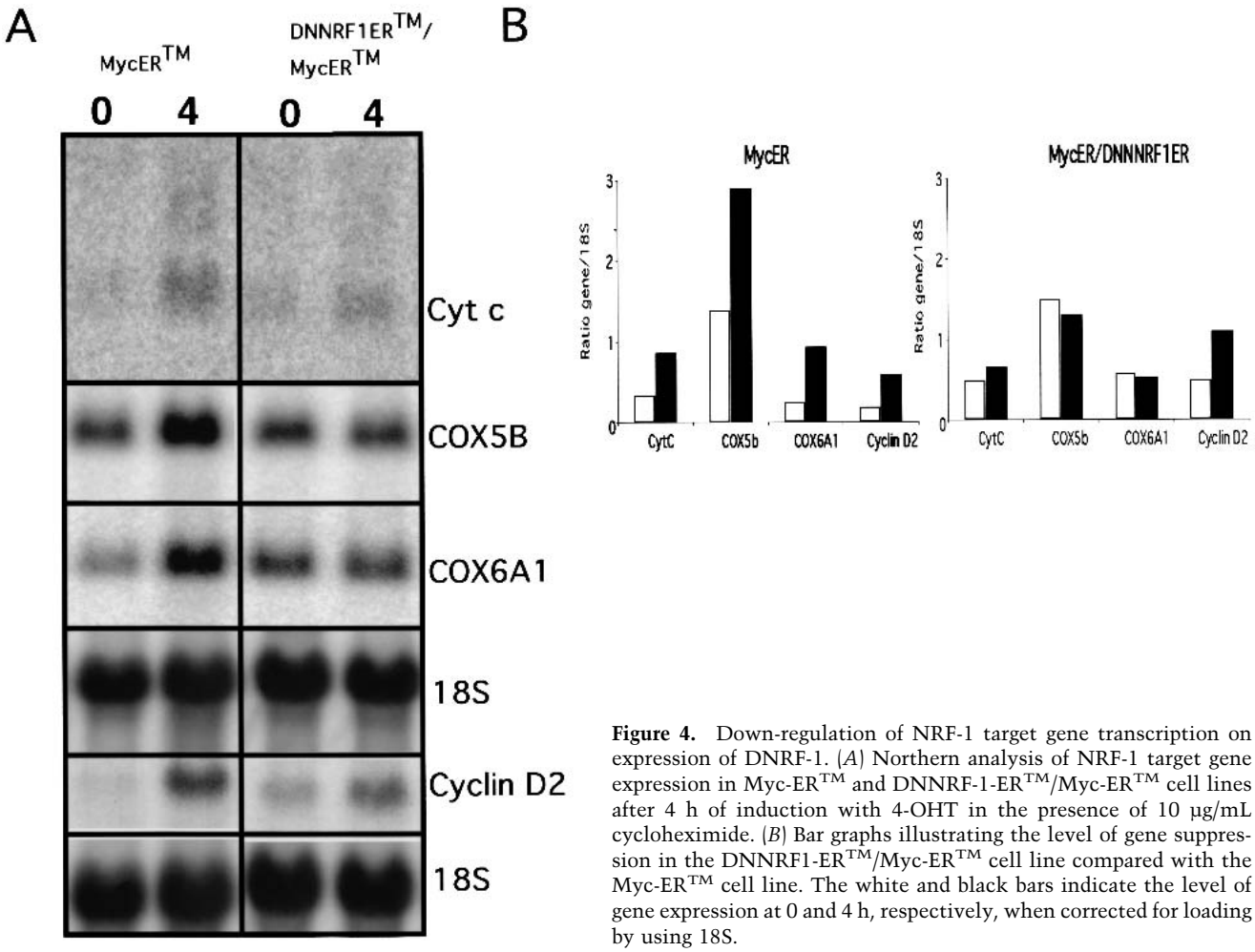


Figure 4. Down-regulation of NRF-1 target gene transcription on expression of DNRF-1. (*A*) Northern analysis of NRF-1 target gene expression in Myc-ER^{TM} and DNNRF-1-ER^{TM}/Myc-ER^{TM} cell lines after 4 h of induction with 4-OHT in the presence of 10 μg/mL cycloheximide. (*B*) Bar graphs illustrating the level of gene suppression in the DNNRF1-ER^{TM}/Myc-ER^{TM} cell line compared with the Myc-ER^{TM} cell line. The white and black bars indicate the level of gene expression at 0 and 4 h, respectively, when corrected for loading by using 18S.

This was in contrast to mitochondria in the DNNRF-1-ER™/Myc-ER™ cell lines, which were enlarged, highly electron dense with obvious cristae, distinctly unusual shapes, and intact membranes (Fig. 6B). The size, shape, and electron density of the mitochondria in DNNRF-1-ER™/Myc-ER™ cells suggest that there has been an increase in mitochondrial biogenesis. To test if these ultrastructural changes were related to differences in mitochondrial function, we performed cytochrome c oxidase (COX) histochemical staining, which allows the analysis of COX activity. When compared with the vector control under serum-deprived conditions, both the Myc-ER™ and DNNRF-1-ER™/Myc-ER™ cell lines showed an increase in COX staining (Fig. 6A). The COX staining in Myc-ER™ cells was very heterogeneous, with a cell population showing both high and low COX staining, whereas COX staining in the DNNRF-1-ER™/Myc-ER™ line was uniformly high.

Discussion

Overexpression of c-myc *leads to dysregulation of genes involved in mitochondrial biogenesis*

A role for c-Myc in cellular metabolism has been recently proposed, owing to the ability of c-Myc to transactivate several glycolytic enzymes (Dang 1999; Osthus et al. 2000). The current study demonstrates that c-Myc is capable of up-regulating the expression of a number of NRF-1 target genes involved in cellular respiration—including cytochrome c, COX5b, and COX6A1—and may thus play a role in the control of oxidative phosphorylation (OXPHOS). Detailed time-course studies in Myc-ER™ cells indicate that cytochrome c is induced twofold when compared to vector controls at 8 h. These levels of induction are comparable to the twofold induction normally found for c-Myc target genes (for review, see Cole and McMahon 1999). c-Myc was also shown to induce transcriptional up-regulation of cytochrome c, as protein levels were increased when compared to the vector control.

Cytochrome c was also found to be a physiological target of c-Myc by analysis of *c-myc*$^{-/-}$ cells. In serum stimulation experiments, a lag in cytochrome c transcription was clearly evident in the *c-myc*$^{-/-}$ line when compared with the wild-type and *c-myc* reconstituted lines. Using ChIP assays, we also demonstrated that c-Myc binds to cytochrome c on serum stimulation, resulting in a 12-fold increase in association of Myc with the target site in the cytochrome c promoter after 4 h. In normal fibroblasts, cytochrome c is known to be induced within 3 h, and this activation is mediated by NRF-1 and CREB (Herzig et al. 2000). Initially, the NRF-1 binding site may not be occupied, as maximal phosphorylation of NRF-1 occurs at 3–6 h after serum addition (Herzig et al. 2000). Our results suggest that c-Myc may occupy this site in the early stages of serum induction, as c-Myc is an immediate-early gene, with increases in transcription of 10- to 20-fold in the first hour after serum addition (Dean et al. 1986).

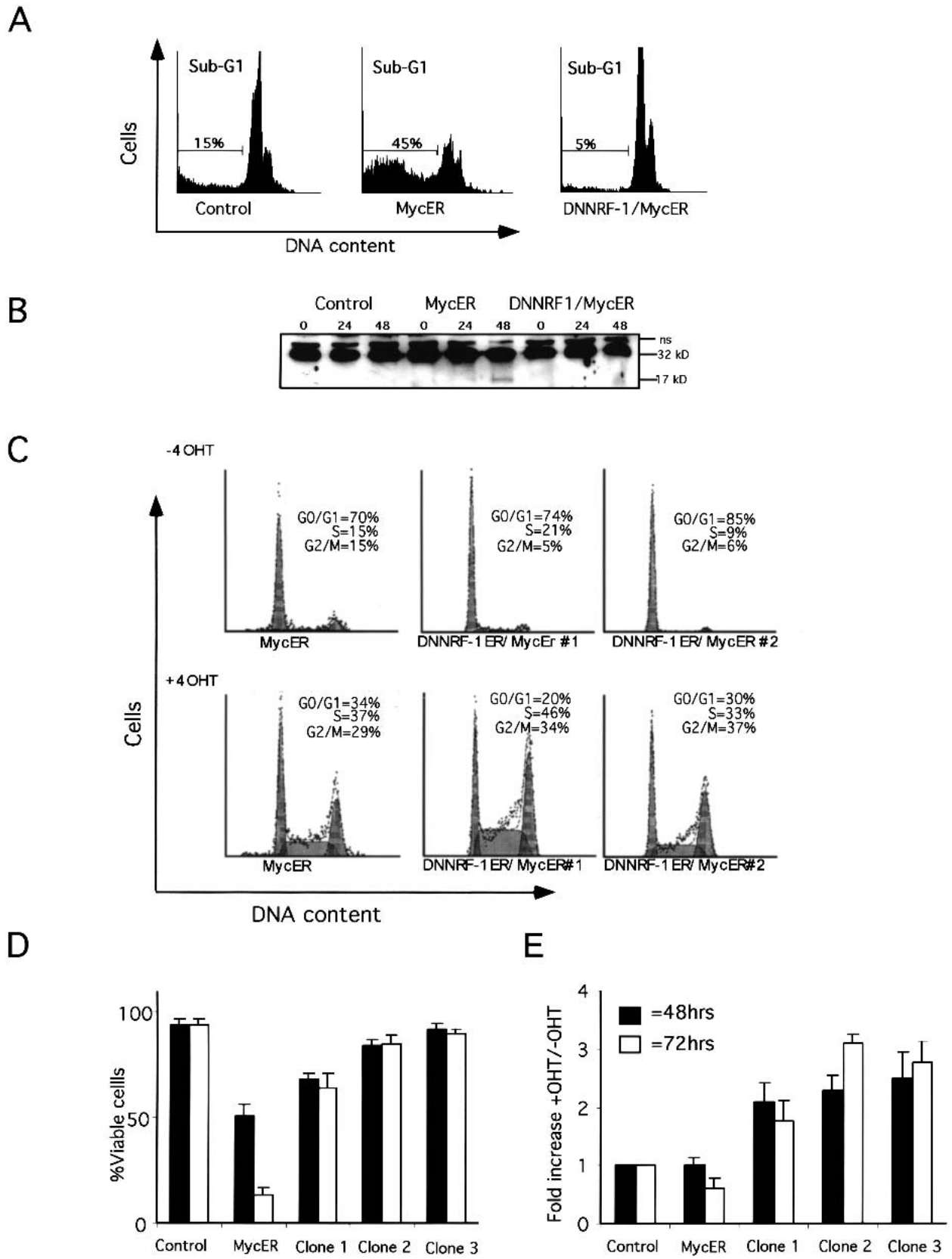
The NRF-1 binding site in the cytochrome c promoter was shown to be required for Myc:MAX binding by EMSA and transient reporter assays. Our data also demonstrate that Myc occupies this site in vivo. Under physiological conditions, the occupancy of an NRF-1/Myc:MAX site by c-Myc or NRF-1 may depend on phosphorylation status, relative affinity, and concentration, and further studies will be needed to address this issue.

The up-regulation of mitochondrial genes by c-Myc has been previously noted in microarray experiments (Coller et al. 1999; Guo et al. 2000), but this is the first confirmation that the gene cytochrome c, which is required for c-Myc-induced apoptosis (Juin et al. 2000) and OXPHOS (Li et al. 2000), is a direct target of c-Myc.

Selective transactivation of a subset of genes involved in mitochondrial biogenesis results in apoptosis

NRF-1 is one of a number of transcription factors involved in regulating genes required for mitochondrial respiration and biogenesis (for review, see Scarpulla, 2002). NRF-1 is particularly important in cell growth and development, as homozygous knockouts of this gene are embryonic lethals (Huo and Scarpulla 2001). Functional NRF-1 sites have been found in genes encoding at least one subunit in each of the respiratory complexes III, IV, and V (Chau et al. 1992; Virbasius et al. 1993). In our efforts to elucidate the possible relationship between c-Myc-induced apoptosis and the expression of NRF-1 target genes, we produced NIH3T3 cell lines expressing NRF-1-ER™. The cell lines produced were shown to up-regulate the genes *cytochrome c* and *mtTFA*, both of which are known functional target genes of NRF-1 (Evans and Scarpulla 1989; Virabsius and Scarpulla 1994), and NRF-1 overexpression also resulted in translational up-regulation of cytochrome c. Furthermore, as observed with expression of c-Myc, the expression of NRF-1-ER™-sensitized cells to apoptosis on serum depletion. This apoptosis was rapid, occurring within 24 h of 4-OHT addition. Electron microscopy studies revealed enlarged, electron-dense mitochondria, disruption of the mitochondrial membrane, nuclear fragmentation, and membrane blebbing. The distinct electron-dense mitochondrial phenotype and increase in mitochondrial size seen in NRF-1-overexpressing cells suggests extensive import of mitochondrial proteins, which may have lead to the disruption of the mitochondrial membrane. These results establish a link between apoptosis and the selective activation of NRF-1 genes. The induction of apoptosis in serum-deprived cells indicates that the continued expression of NRF-1 under these conditions is detrimental to cell survival.

The characteristic bursting of mitochondria seen in NRF-1-overexpressing cells in low serum was not present in lines in which the antiapoptotic protein Bcl-2 was introduced. Similarly, Bcl-2 inhibited NRF-1-induced apoptosis and accumulation of sub-G1 cells. The ability of Bcl-2 to inhibit both NRF-1 and c-Myc induced apoptosis is consistent with the induction of shared



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apoptotic signaling pathways, resulting in mitochondria dysfunction and cell death.

Inhibition of NRF-1 target gene expression inhibits c-Myc-induced apoptosis but not c-Myc-induced proliferation

The establishment of a link between the activation of NRF-1 target genes and apoptosis suggests that NRF-1 target genes could potentially mediate c-Myc-induced apoptosis. We tested if the transactivation of these genes is required for c-Myc-induced apoptosis by using a truncated form of NRF-1 lacking the transactivation domain (DNNRF-1). This DNNRF-1 has been shown to act as a dominant-negative mutant in transient transfections (Wu et al. 2000). To determine if the binding of this mutant to NRF-1 target sites would block Myc-induced apoptosis, an ER fusion version of this gene was introduced into Myc-ER cells. Cells were induced to coactivate DNNRF-1-ER^{TM} and Myc-ER^{TM} by the addition of 4-OHT and compared with cells containing activated Myc-ER^{TM} in low serum. In these studies, minimal levels of apoptosis were observed in DNNRF-1-ER^{TM}/Myc-ER^{TM} cells compared with Myc-ER^{TM}. However, proliferation was not affected, as there was no significant drop in the percentage of cells in S phase, and cell numbers increased. Furthermore, caspase-3 cleavage was inhibited, and analysis of these cells by electron microscopy indicated that there were significant ultrastructural differences. Myc-ER^{TM} cells at 48 h displayed all the typical features of apoptotic cells; in contrast, the majority of cells in the DNNRF-1-ER^{TM}/Myc-ER^{TM} line showed no nuclear fragmentation or vacuolation. They did show distinctive mitochondrial morphologies with an electron-dense matrix, obvious convoluted cristae, and enlarged irregular shapes. These changes are potentially related to differences in metabolism (see below), and similar ultrastructural changes have been noted in cells treated with thyroid hormone (Jakovicic et al. 1978).

The inhibition of apoptosis by DNNRF-1 was associated with a reduction in the transcript levels of a series of genes including cytochrome c, COX5b, and COX6A1. All of these genes have functional NRF-1 binding sites (Evans and Scarpulla 1989; Virbasius et al. 1993; Wong-Riley et al. 2000) and are necessary for the activity of respiratory complex IV (COX; for review, see Grossman and Lomax 1997; Kadenbach et al. 2000). COX consists of 10 nuclear and three mitochondrially derived subunits (for review, see Capaldi, 1990), and COX histochemical assays can provide a measure of mitochondrial respiration (D'Herde et al. 2000). When we evaluated the Myc-ER^{TM} and DNNRF-1-ER^{TM}/Myc-ER^{TM} cells line by COX histochemistry, both lines had higher COX activity than that of the vector control line. The DNNRF-1-ER^{TM}/Myc-ER^{TM} lines showed uniform levels of COX activity in all cells; in contrast, the cell population in the Myc-ER^{TM} line had both high and low levels of COX activity. Similar cell population mosaicism, correlated to the release of cytochrome c and loss of respiratory capacity, has been reported to arise during FAS-mediated apoptosis (Hajek et al. 2001). The uniform COX activity present in the DNNRF-1-ER^{TM}/Myc-ER^{TM} cell line suggests that DNNRF-1-ER^{TM} maintains mitochondrial function and respiratory capacity by limiting the transactivation of cytochrome c and other NRF-1 target genes. Thus, the molecular events underlying the mitochondrial dysfunction present in Myc-ER^{TM} cells may be explained by the inappropriate expression of a subset of NRF-1 target genes involved in mitochondrial biogenesis. This would lead to an imbalance of nuclear-encoded and mtDNA-encoded subunits, resulting in the production of misassembled and defective OXPHOS complexes. Such defective complexes occur in mitochondrial diseases and can lead to cell death, owing to release of reactive oxygen species and a diminished level of respiration (Poyton 1998). We propose that a dysfunction in the assembly of mitochondrial protein complexes may act as a catalyst for mitochondrial membrane rupture and the release of apoptotic factors involved in c-Myc-induced apoptosis. This hypothesis is corroborated by recent data from studies of mitochondrial encephalomyopathy, which indicate that cells with low levels of COX activity show high levels of apoptosis (Mirabella et al. 2000). Further analysis of the molecular events underlying mitochondrial dysfunction in Myc-ER^{TM} cells may help resolve how the release of apoptotic factors is initiated.

Although our results show that DNNRF-1 inhibits c-Myc-induced apoptosis, there was no impact on the proliferative function of c-Myc. This result is in contrast to other studies on the transcriptional repressor MAD1, which competes with c-Myc for MAX binding (Bejarano et al. 2000; Gehring et al. 2000). In these reports, apoptosis was inhibited, but the proliferative function of c-Myc was compromised, as the use of MAD1 targeted the repression of a broad array of c-Myc target genes, owing to its potential ability to bind both canonical and non-

Figure 5. Introduction of DNNRF1 into Myc-ER^{TM} cells inhibits c-Myc-induced apoptosis but maintains cell cycle induction and cell proliferation. (*A*) PI FACS analysis of vector control, Myc-ER^{TM}, and a DNNRF-1-ER^{TM}/Myc-ER^{TM} cell line, indicating the percentage of sub-G1 cells in the population 48 h after 4-OHT addition. A minimum of 10^5 cells were analyzed in triplicate for each sample, and these results are representative of three separate experiments. (*B*) Western analysis using caspase-3 antibody to detect cleavage of caspase 3. (*C*) PI FACS analysis of the cell cycle in the Myc-ER^{TM} cell line and two representative DNNRF-1-ER^{TM}/Myc-ER^{TM} clones (#1 and #2) in serum-deprived cells in the presence and absence of 4-OHT. Cells were serum depleted in 0.5% serum for 48 h prior to culture in the presence or absence of 4-OHT for 24 h. A minimum of 10^5 cells were analyzed in triplicate for each sample, and these results are representative of three separate experiments. (*D*) Viability analysis at 48 and 72 h after 4-OHT addition. (*E*) Proliferation analysis at 48 and 72 h after 4-OHT addition. Data are expressed as the relative increase in cell proliferation on the addition of 4-OHT compared with cells maintained in the absence of 4-OHT.

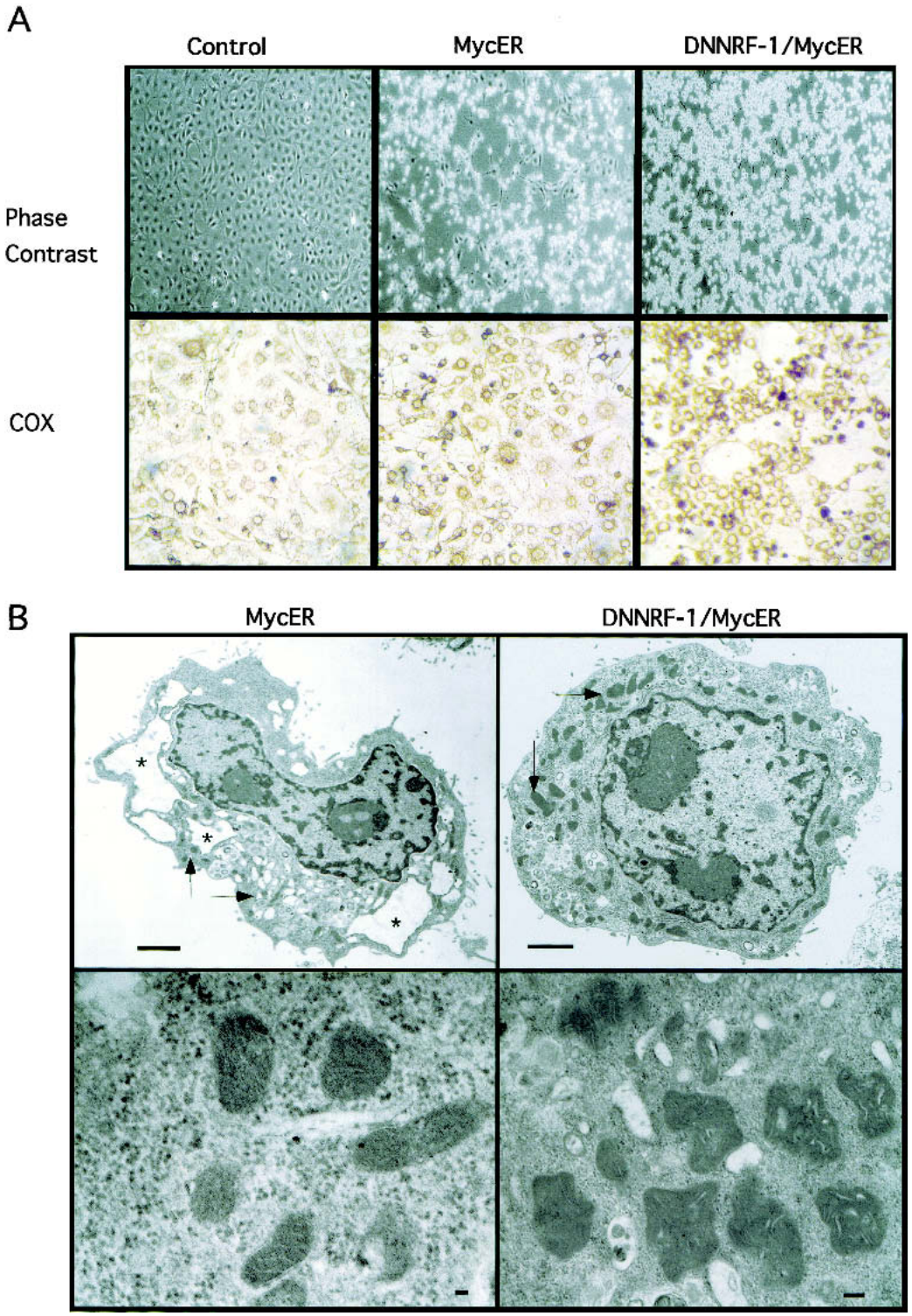

Figure 6. Cellular morphology, ultrastructural, and respiratory changes induced on activation of DNNRF-1 in MycER^{TM} cells. (*A*) Phase microscopy and COX histochemistry of cells from control, Myc-ER^{TM}, and a DNNRF-1-ER^{TM}/Myc-ER^{TM} clone 48 h after addition of 4-OHT in 0.5% serum. Cells had been serum starved for 48 h prior to 4-OHT addition. (*B*) Electron micrographs of Myc-ER^{TM} and DNNRF-1-ER^{TM}/MycER-ER^{TM} cells 48 h after 4-OHT addition. The arrows point to mitochondria; asterisks highlight the dilated ER. High-power magnification of mitochondria in Myc-ER^{TM} cells and DNNRF1-ER^{TM}/Myc-ER^{TM} cells illustrates the distinct morphologies of these mitochondria in 0.5% serum. Note that the Myc-ER^{TM} mitochondria have been magnified 2.5× to allow comparison with the mitochondria in the DNNRF1-ER^{TM}/Myc-ER^{TM} cell line. Bars: *upper* panels, 2 μm; *lower* panels, 200 nm.

canonical Myc:MAX binding site. The NRF-1 binding site is palindromic (T/C)GCGCA(T/C)GCGC(A/G), and the NRF-1 protein makes major groove contacts through alternating GC base pairs. Furthermore, in vivo studies have shown that stable, high-affinity binding requires a tandem direct repeat of the T/CGCGCA motif (Virbasius et al. 1993). This would suggest that DNNRF-1 would not bind a canonical Myc:MAX site. Our evidence demonstrates no repression by DNNRF-1 of the genes cyclin D2 or CAD, which have canonical Myc:MAX binding sites (Boyd and Farnham 1997; Bouchard et al. 1999). This implies that the target genes shared by NRF-1 and c-Myc may be limited to a subset of genes containing the noncanonical Myc:MAX binding site CA(T/C)GCG, which has adjacent nucleotides that are compatible with both NRF-1 and Myc:Max binding. Studies are ongoing to elucidate these shared gene targets and confirm the selectivity of binding.

The fact that DNNRF-1 reduces apoptosis suggests that up-regulation of NRF-1 target genes by c-Myc is responsible for the activation of the mitochondrial apoptotic signaling pathway. It is clear that these genes play dual roles; for example, cytochrome c is essential for both cellular proliferation and apoptosis (Li et al. 2000). Hence, the paradox of the dual ability of c-Myc to induce proliferation and apoptosis as initially proposed (Evan and Littlewood 1993) might be resolved by a more central paradox in the form of c-Myc target genes that have dual functions in both apoptosis and cellular proliferation. We propose that the cell requires a tightly controlled balance of these genes to survive. It is the disruption of this balance, by selective c-Myc overexpression, which leads to the cells demise (Fig. 7).

Material and methods

Cell culture

NIH3T3 and Rat1 cells were grown in phenol-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.3% penicillin and streptomycin. Cultures were grown at 37°C in an atmosphere of 5% CO_2. The cell line NIH3T3 Myc-ER^{TM} was provided by Martin Eilers (Institute of Molecular Biology and Tumor Research, Marburg, Germany). The NIH3T3 vector control cell line was produced by transduction of NIH3T3 cells as described below. The TGR-1 *c-myc*$^{+/+}$ and c-*myc*$^{-/-}$ cells were obtained from John Sedivy, Yale University, New Haven, CT (Mateyak et al. 1997), and the lines KO empty vector, KOMyc-ER^{TM} were provided by Carla Grandori, Fred Hutchinson Cancer Research Center, Seattle, WA (Hirst and Grandori 2000).

Electromobility shift assays

The 50 base pairs of dsDNA encompassing the NRF-1 binding site from the cytochrome c promoter TGCTAGGCCGCATGCGCGCCCTTGCTAGCGCTCCCAATCCTGGAGCCAATGA were used in these assays, and an NRF-1 mutant, which differed only in the core NRF-1 binding region (CTCATTAG), was used to test specific binding of Myc:MAX to this site. This was tested by EMSA as described (Grandori et al. 1996). Briefly, the DNA was treated with calf intestinal phosphatase, end-labeled with T4 kinase and high-specific activity [^{32}P]γ-ATP (ICN). Labeled DNA was incubated with recombinant Myc (25 ng; Carla Grandori, FHCRC), MAX (7.5 ng; Yuzuru Shiio, FHCRC), or Myc:MAX (25 : 7.5 ng). Competition analysis was undertaken with unlabeled competitors, including wild-type and mutated cytochrome c dsDNA oligonucleotides, an dsDNA oligonucleotide containing a consensus Myc:MAX binding site (CCCCACCACGTGGTGCCTGACACGTG) and an identical sequence with a mutated Myc:MAX binding site (CCTTGAGG; Amir Oryan, Fred Hutchinson Cancer Research Center). Binding reactions were carried out in 10 µL HMO buffer (25 mM HEPES at pH 7.5, 50 mM KCL, 5 mM $MgCl_2$, 0.5 mM EDTA, 5% glycerol, 10 mM dithiothreitol, 0.1% NP40, 0.5 mg/mL BSA) in the presence of salmon sperm DNA. Products were analyzed on a 4% HEPES-acrylamide nondenaturing gel.

ChIP and PCR amplification

Formaldehyde cross-linking and chromatin immunoprecipitation were carried out according to the protocol of Orlando et al. (1997) with minor modifications. Quiescent and serum-induced cells (2×10^8) were fixed in 150 mL of DMEM with 1% formaldehyde for 10 min at room temperature. Sonicated cross-linked chromatin was adjusted to 1× RIPA buffer before immunoprecipitation by adding 2× RIPA buffer. Rabbit polyclonal antibodies (Santa Cruz; anti-Myc sc-764) were used to immunoprecipitate chromatin. Immunoprecipitated samples or no antibody controls were resuspended in 25 µL of TE, and 1.5 µL from each sample was analyzed by PCR. Each PCR reaction contained 1.5 µL of DNA sample, 0.5 µM of each primer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 1× Taq buffer, 1.25 U of Taq DNA polymerase

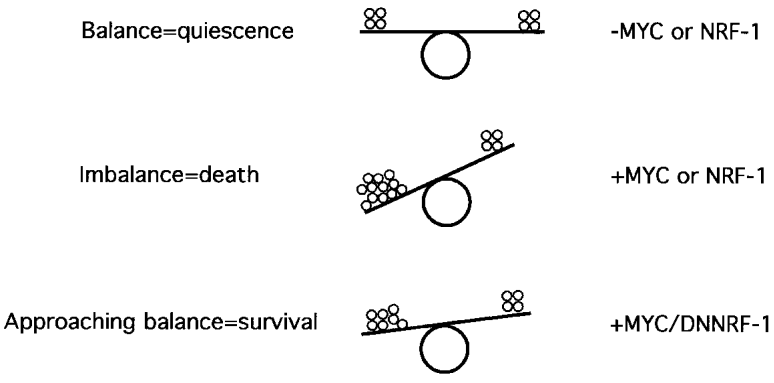

Figure 7. A model for the role of balanced mitochondrial gene expression in the maintenance of cell viability. In the serum-starved cell, quiescence is achieved by creating a balanced expression of genes. On the induction of the transcription factors c-Myc or NRF-1, this balance is disrupted due to the inappropriate expression of a subset of target genes required to maintain mitochondrial function. This leads to mitochondrial dysfunction and death. On the addition of DNNRF-1, a selected subset of genes, which are involved in mitochondrial dysfunction, are down-regulated, allowing the cell to survive. As not all c-Myc target genes are down-regulated, the cell continues to proliferate in the presence of dual signals, which allow survival and proliferation.

(Invitrogen), and 0.1 µL of [^{32}P] dCTP (NEN). DNA isolated from nonimmunoprecipitated chromatin samples was diluted 200-fold, and 0.5 µL was used for PCR. PCR reactions were performed in parallel under conditions of linear amplification in a Perkin Elmer 9600 thermocycler for 30 cycles by using identical temperature profiles for all primer pairs. One third of the reaction was subjected to electrophoresis on a 5% polyacrylamide gel and quantified with a PhosphoImager (Molecular Dynamics) and Image Quant software.

Primers

The following primer pairs were used to amplify rat genomic sequences: rat cytochrome c (GenBank accession no. M19870) CytC-F (GCTAGTCAGGGAATCTAGACCAAGC) and CytC-R (TCATTGGCTCCAGGATTGG), amplicon of 348 bp containing a single NRF-1 binding site; rat albumin primer pair Alb-F (TTCACGGCCTCTACATAG) and Alb-R (GTTTGCCATCT GAAGCTC); and COX5b (GenBank accession no. D10951) primer pair COX5b-F (CCTAACTGAGAGGGACTTCG) and COX5B-R (CGTTGGTAGGTGTCTAACAACG), which produces an amplicon of 239 bp with a single NRF1 binding site.

Transcriptional activation assays

NIH3T3 cells were plated at a density of 4×10^4 cells per well in 24-well plates and grown for 24 h before transfection by the calcium phosphate method. Both the wild-type and NRF-1 mutant cytochrome c promoter constructs have been described (Evans and Scarpulla 1989; Xia et al. 1997). Both consist of a 1.1-kb fragment, which includes the 5′-flanking region and the first intron of rat cytochrome c, inserted upstream of the luciferase gene in the vector pGL3 (Promega), and are named pGL3RC4/-326 and pGL3RC4/-326 NRF-1 mut, respectively. The c-Myc transactivating construct consisted of a 2-kb fragment of *c-myc* inserted in the empty vector pRcCMV (Invitrogen). In all transfections, the vector pRSV β-gal was cotransfected as an internal control for transfection efficiency. In all experiments, the data were evaluated relative to luciferase expression in cells transfected with empty vector (pRcCMV); this vector was also used to equalize the amount of DNA used in all transfections, which was uniformly 100 ng. In experiments to test the requirement for the NRF-1 site for c-Myc transactivation, the transfection mix included 0.05 ng pRSV β-gal, 0.5 ng pGL3RC4/-326 or pGL3RC4/-326 NRF-1 mut, and 15 ng of pRcCMVMyc, with empty vector added to bring the sample DNA to 100 ng. Cells were harvested 24 h after transfection and evaluated for luciferase and β-gal activity as described (Xia et al. 1997).

Northern analysis

Total RNA was isolated by using Triazol (GIBCO-BRL). A total of 15–20 µg of RNA was run on a 1.2% formaldehyde-agarose gel, blotted to Zetaprobe GT nylon membrane (Bio-Rad), and hybridized in ULTRAhyb buffer (Ambion) at 42°C. These blots were then probed with [^{32}P] random primed labeled probes (Ambion, Decaprime kit), including rat cytochrome c (from Richard Scarpulla, Northwestern University, Chicago, IL), mouse mitochondrial transcription factor A (from David Clayton, Howard Hughes Medical Institute, Washington, DC), human NRF-1 (from Richard Scarpulla), COX6A1 (from Margaret Lomax, Medical College of Wisconsin, Milwaukee, WI), COX5b (from Narayan Avadhani, University of Pennsylvania, Philadelphia, PA), and 18S rRNA (Ambion).

Protein labeling and Western analysis

Cells were serum starved for 48 h prior to addition of Trans ^{35}S label (ICN) at 100 µci/mL. Cells were preincubated for 30 min in DMEM without methionine/cysteine (GIBCO), labeled for 2–4 h, and harvested into NP-40 isotonic lysis buffer (Oltvai et al. 1993). After nutation for 30 min and centrifugation for 5 min at 13,000 rpm at 4°C the resultant supernatant was precleared with agarose-conjugated mouse IgG (Santa Cruz). The supernatant was then immunoprecipitated with cytochrome c antibody conjugated to protein G-Sepharose (Amersham) for 90 min. For the control, the supernatant was immunoprecipitated with agarose- conjugated mouse IgG. Samples were electrophoresed on a 15% acrylamide gel, fixed, and treated with Amplify (Amersham) prior to drying. For Western analysis, cells were lysed in NP-40 buffer; samples were electrophoresed on a 15% acrylamide gel; and the protein was transferred to nitrocellulose, blocked with 5% Blotto, and incubated overnight with antibodies to caspase 3 (Santa Cruz) or estrogen receptor (Santa Cruz, Sc-542).

Plasmid constructions and transductions

For the construction of NRF-1-ER^{TM}, PCR was used to add a Flag tag (Knappik and Pluckthun 1994) immediately upstream of the ATG of a human NRF-1 cDNA clone (a gift from Richard Scarpulla). PCR was also used to delete the 3′ end to amino acid 407 and add a *Bam*HI site to allow in frame fusion with the estrogen receptor. A *Bam*HI fragment containing this Flag-NRF-1 fragment was inserted in place of the c-*myc Bam*HI fragment in the vector pBabe-puro Myc-ER^{TM} (a gift from Trevor Littlewood, Imperial Cancer Research Institute, London). A similar strategy was used to produce a Flag-tagged DNNRF-1-ER^{TM}. In this case, PCR was used to delete the 3′ end to amino acid 307 and to again add a *Bam*H1 site for in-frame fusion with the estrogen receptor. This *Bam*HI FlagDNNRF-1 fragment was inserted in place of the c-*myc Bam*H1 fragment in the vector pBabe-NeoMyc-ER^{TM} (a gift from Trevor Littlewood) to allow selection with G418. All constructs were confirmed by sequencing. Viral supernatants were generated from these retroviral vectors by transfection into the ecotropic retroviral packaging cell line BOSC23 (Pear et al. 1993). For retroviral transduction, 1×10^6 cells were plated in 10-cm plates and transduced for 24–48 h in the presence of 8 µg/mL polybrene, followed by selection with 5 µg/mL puromycin or 1 mg/mL G418. Puromycin or G418-resistant clones were selected and maintained in phenolred-free DMEM medium.

The production of NRF-1-ER^{TM} lines coexpressing Bcl-2 was accomplished by transfection of the vector pSSVBcl-2 (Hockenbery et al. 1993). G418-resistant clones were selected and maintained in phenol-free DMEM medium. The vector control NIH3T3 line used in all experiments was produced by transduction with viral supernatant harboring the vector pBabePURO (a gift from Trevor Littlewood). Puromycin-resistant clones were selected and maintained in phenol-free DMEM medium.

Transmission electron microscopy

Cells were harvested and resuspended in half-strength Karnovsky's fixative for 4–6 h, rinsed in 0.1 M cacodylate buffer, and postfixed in 1% collidine-buffered osmium tetroxide. Dehydration in graded ethanol and propylene oxide was followed by infiltration and embedding in Epon 812. Sections of 70–90 nm were stained by using saturated, aqueous uranyl acetate and lead tartrate. Photographs were taken by using a transmission electron microscope (100 SX; Jeol Ltd., Tokyo, Japan) operating at 80 kV.

Cell cycle and apoptosis analysis

Propidium iodide (PI) staining of whole cells was analyzed by flow cytometry. A minimum of 10^5 cells were analyzed in triplicate for each sample, and results are representative of three separate experiments. Both adherent and floating cells were collected, washed in PBS supplemented with 1% FCS, and fixed in 80% ethanol at 4°C. After washing with PBS, cells were permeabilized with 0.25% Triton X-100 for 5 min, washed, and stained in PI solution in PBS (10 µg/mL PI, 1 mg/mL DNase-free RNase) for 30 min at 37°C. The DNA fluorescence was measured by using a FACScanR (Becton Dickinson, Bedford, MA), and data analysis for the distribution of cells in sub-G1, G0/G1, S and G2/M was performed by using Multicycle software (Kallioniemi et al. 1994).

Cytochrome oxidase histochemistry

Cells were plated in 96-well plates in DMEM supplemented with 10% serum and grown overnight. The next day, cells were serum-depleted by replacing the medium with DMEM supplemented with 0.5% serum and grown for 48 h. At this time point, cells were supplemented with a further 100 µL of DMEM containing 0.5% serum ±100 nM 4-OHT and were left to grow for a further 24–48 h. COX histochemical analysis was undertaken at 0, 24, and 48 h. The medium was removed and replaced with 100 µL of a COX assay buffer (2 mg/mL catalase, 1 mg/mL cytochrome c, 0.5 mg/mL 3,3′-diaminobenzidine tetrahydrochloride, and 74 mg/mL sucrose in 0.05 M sodium phosphate buffer at pH 7.4; Seligman et al. 1968). Cells were incubated for 1 h at 37°C prior to analysis and photography with a Nikon inverted scope.

Acknowledgments

We would like to say a special thank you to Carla Grandori and Richard Scarpulla for their helpful discussions throughout the course of this work and for sharing their respective knowledge of Myc and NRF-1. We gratefully appreciate the many gifts of plasmids, antibodies, and cell lines from Richard Scarpulla, David Clayton, John Sedivy, Narayan Avadhani, Maragret Lomax, Carla Grandori, Trevor Littlewood, Gerard Evan, Don Bergstrom, and Martin Eilers. We thank Yuzuru Shiio for recombinant MAX, Carla Grandori for recombinant Myc, and Amir Oryan for E-box oligonucleotides.. We thank all those in the Electron Microscopy, Flow Cytometry, and Scientific Imaging facilities. Our thanks to Tim Knight and Deborah Anderson for help with scientific imaging and Judy Groombridge, Liz Caldwell, and Franque Remington for assistance with EM analysis. For their critical reading of this manuscript and useful discussions, we would like to thank Carla Grandori and John Lamb. We also gratefully acknowledge discussions with Robert Eisenman during the course of this work. These studies were supported by NIH grant DK56761-01 to D.H., and F.M. is a recipient of an NRSA fellowship from the NIH. This work is dedicated to the memory of Mairin, Dairine, and Sean O'Connor.

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