Activity of the human cytochrome c_1 promoter is modulated by E2F

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The human cytochrome c_1 promoter is strongly activated in transfected *Drosophila* SL2 cells expressing exogenous human E2F1. Transfection-deletion experiments, DNase I protection by E2F1 and gel mobility-shift experiments locate E2F1 activation sites to two regions on either side of the transcription start site. Deletion of either region prevents E2F1 activation in transfected SL2 cells, suggesting a co-operative interaction between them. E2F6, a member of the E2F family that lacks transactivation domains but contains specific suppressor domains, inhibits cytochrome c_1 promoter activity when co-transfected into HeLa cells, indicating that the E2F proteins modulate the cytochrome c_1 promoter in mammalian cells. However, E2F is not a general regulator of oxidative phosphorylation genes since three additional nuclear-encoded mitochondrial genes were unaffected by E2F1 or E2F6.

Key words: *Drosophila*, mitochondria, oxidative phosphorylation, Sp1, transcription.

INTRODUCTION

An increase in mitochondrial mass during periods of cell growth and division must ultimately require accumulation of all the 600 or so polypeptides that make up the organelle. Little is known about the mechanism(s) by which the bulk expression of these polypeptides is regulated [1–4]. However, a set of mitochondrial genes controlled by the nuclear respiratory factors (NRFs, reviewed in [5]) has recently been shown to come under control of the thermogenic co-activator, PGC-1 (PPARγ co-activator-1) [6,7].

During the course of studies to search the promoters of nuclear-encoded mitochondrial genes for features that might direct such global regulation, we observed [8] that the cytochrome c_1 promoter is organized similarly to the promoters of two E2F-dependent, growth-regulated genes; mouse dihydrofolate reductase (DHFR) [9] and thymidine kinase [10]. Like these TATA-less promoters, the human cytochrome c_1 promoter is activated through proximal Sp1 elements. It also contains an initiator region at the transcription start site [8], which contains, or is flanked by, elements that closely match the E2F consensus element [11]. Since growth-dependent expression of DHFR and thymidine kinase is strongly dependent on E2F1, and in view of the organization of the cytochrome c_1 promoter, we studied the effects of E2F on the cytochrome c_1 promoter. The results show that the cytochrome c_1 promoter is modulated by both E2F1 and E2F6; being activated by the former and suppressed by the latter. However, the E2F proteins are not general effectors of oxidative phosphorylation (OXPHOS) gene expression since the activities of three additional OXPHOS promoters were unaffected by these factors.

EXPERIMENTAL

Chloramphenicol acetyltransferase (CAT) reporter constructs containing human cytochrome c_1 promoter fragments were prepared as described previously [8]. Oligonucleotides covering the two E2F-like sites in the cytochrome c_1 promoter region were synthesized: cytochrome c_1 ($-13/+12$), 5′-CTCCCCGGCTT-TCGCGAGGTTTTGA-3′, and cytochrome c_1 ($+6/+30$), 5′-GTTTTGACTCTCGTGGCGCCCCAGG-3′. Oligonucleotides

containing wild-type E2F (E2F-Wt), 5′-ATTTAAGTTTCG-CGCCCTTTCTCAA-3′, and mutated E2F (E2F-Mut; underlined CG → AT) sequences were purchased from Santa Cruz Biotechnology.

Clones covering the promoter regions of human adenine nucleotide translocator 2 (ANT2) and F_1-ATPase β-subunit genes were prepared as described in [12]. The human mitochondrial transcription factor A (mtTFA) promoter region (nt -634 to $+92$, numbered relative to the transcription start site [13]) was amplified from human peripheral lymphocyte DNA by PCR using 5′ and 3′ primers that included *Pst*I and *Xba*I sites, respectively. Amplified DNA was cloned into the polylinker of pCATbasic (Promega).
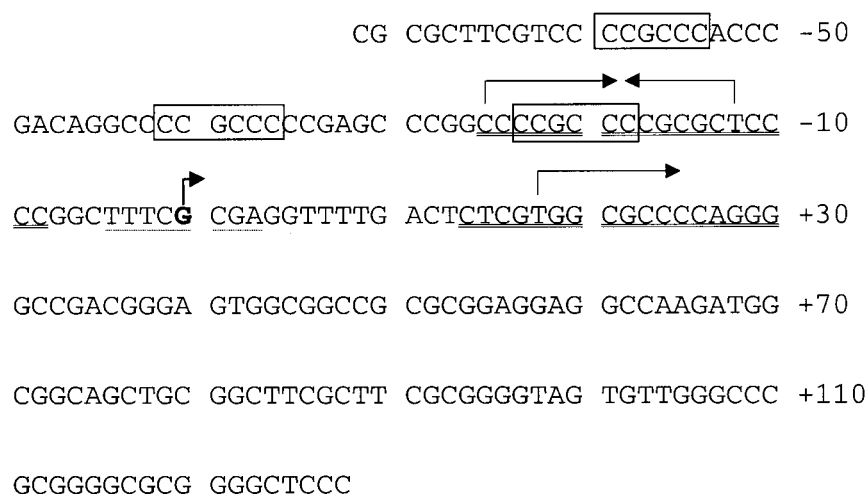
Drosophila SL2 cells were grown at 25 °C in Schneider medium, and Hela cells were grown at 37 °C in Dulbecco's modified Eagle's medium. Both media were fortified with 10% fetal bovine serum (Gibco-BRL), 2 mM glutamine, 50 units of penicillin and 50 μg/ml streptomycin. Actively growing cells (5×10^6 cells) were transfected using the calcium phosphate/DNA co-precipitation procedure [14]. SL2 cells were transfected with 5 μg of a promoter/CAT reporter plasmid, and 1 or 2.5 μg of the human Sp1 (pPac-Sp1) [15] or human E2F1 (pPac-E2F1) [16] expression vectors, respectively. Transfection efficiency was determined by co-transfection of 0.5 μg of pPac-β-Gal (a gift from Y. Yngström, Stockholm University, Stockholm, Sweden). HeLa cells were transfected with 10 μg of reporter plasmid and 1 μg of pCDNA3-HA-E2F6 [17] expressing human E2F6. Transfection efficiency was determined by co-transfection of 1 μg of pSV-β-Gal (Promega). Total DNA in the transfection media was adjusted to a constant amount with pGEM-3Zf (Promega). Transfections were carried out in triplicate for each experimental point. Cells were collected after 48 h, CAT and β-galactosidase activities were measured [8], and the ratio CAT/β-Gal was calculated. The ratio in the absence of a co-expressed transcription factor was used as the baseline value from which fold induction was calculated in SL2 cells.

Electrophoretic mobility-shift assays were performed as described in [8]. The DNase I protection assay was performed as in [8] using the following promoter fragments: cytochrome c_1 ($-72/+128$) [8], ANT2 ($-235/+46$) [12,15], F_1-ATPase

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              CG CGCTTCGTCC CCGCCC ACCC -50

GACAGGC CC GCCC CCGAGC CCGG CC CCGC CC CGCGCTCC -10

CC GGCTTTC G CGAGGTTTTG ACT CTCGTGG CGCCCCAGGG +30

GCCGACGGGA GTGGCGGCCG CGCGGAGGAG GCCAAGATGG +70

CGGCAGCTGC GGCTTCGCTT CGCGGGGTAG TGTTGGGCCC +110

GCGGGGCGCG GGGCTCCC
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Figure 1   The proximal region of human cytochrome c_1 region

The proximal region of the human cytochrome c_1 promoter is shown. The short arrow marks the transcription start site ($+1$). The three boxed sequences represent Sp1 core elements required for promoter activation [8]. Double-underlined sequences are protected by purified human E2F1 in the DNase I protection assay, and long arrows represent E2F consensus sites or near-consensus sites. Single-underlined nucleotides represent an E2F-like sequence within the initiator region that is not protected by E2F1 in the DNase I assay.

	ACTIVATION BY (fold induction)		
	E2F1	SP1	SP1+E2F1
−1339/+128	33.3 ± 11.1	49.5 ± 19	45.4 ± 27.6
−72/+128	17.6 ± 3.4	20.6 ± 4.1	17.7 ± 2.2
−35/+128	20.6 ± 9.8	24.3 ± 8.2	23.6 ± 6.1
−13/+128	3.1 ± 0.3	1.6 ± 0.4	1.9 ± 1.2
−72/+128	20.2 ± 9.9	22.4 ± 9.7	18.4 ± 7.7
−72/+29	17.1 ± 3.6	19.4 ± 3.1	21.4 ± 8.3
−72/+18	9.6 ± 3.4	16.4 ± 5.6	12.5 ± 4.1
−72/+1	2.8 ± 0.7	17.6 ± 1.8	14.8 ± 1.8

Figure 2   The E2F1 activation site is located in the proximal region of the human cytochrome c_1 promoter

SL2 cells were transfected with 5.0 μg of different 5'-deletion contructs (upper panel) or 3'-deletion constructs (lower panel) from the cytochrome c_1 promoter together with 2.5 μg of the human E2F1 expression vector (pPac-E2F1), 1 μg of the human Sp1 expression vector (pPac-Sp1), or both. Transcription start sites (arrows), putative or demonstrated Sp1 sites (black ellipses) and sites protected by purified E2F1 (□) are indicated. The downstream E2F protected site is depicted as a double box in order to illustrate the fact that clone $−72/+18$ includes only the 5' half of the protected region. Fold activation was calculated relative to the expression of the respective pCAT-CC1 construct in the absence of any mammalian transcription factors. All values represent the means ± S.D. for at least three independent transfection experiments, in which each data point was collected in triplicate.

β-subunit ($-40/+206$) [18] and mtTFA ($-79/+92$) [19]. Radiolabelled probes for the upper and lower strands were prepared by PCR using 5'-end-labelled oligonucleotides corresponding to nt 2230–2208 (M13 primer) or nt 2307–2285 of pCATbasic. Recombinant, purified human Sp1 was from Promega. Recombinant human E2F1 was isolated as described below. A footprint unit of Sp1 or E2F1 was defined as the lowest amount of protein giving full protection in the DNase I protection assay (Promega).

For expression of E2F1, *Escherichia coli* BL 21 cells transformed with a plasmid containing a glutathione S-transferase (GST)-E2F1 construct [10] were grown at 37 °C in $2 \times$ YT medium (1.6 % tryptone/1 % yeast extract/0.5 % NaCl, pH 7) supplemented with 100 μg/ml ampicillin until the D_{550} value reached 0.7. Cells were induced with 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 30 °C. GST-E2F1 was isolated on glutathione-Sepharose 4B columns (Pharmacia) as described in [20], and its binding capacity was tested in the electrophoretic mobility-shift assay using consensus wild-type E2F (E2F-Wt) and mutated E2F (E2F-Mut) oligonucleotides as probes.

RESULTS

The cytochrome c_1 promoter contains three putative E2F elements (Figure 1). To test if these elements can confer E2F modulation on the cytochrome c_1 promoter, *Drosophila* SL2 cells were transfected with phCC1 (the promoter region of the human cytochrome c_1 gene, encompassing nt $-1339/+128$) [8] and a vector expressing human E2F1 [16]. Figure 2 shows that CAT expression driven by the cytochrome c_1 promoter is induced to nearly the same extent by E2F1 and Sp1, the latter of which was shown previously to be the major activator of the promoter [8]. Little or no CAT activity was measured in the absence of expressed E2F1, indicating that the *Drosophila* E2F homologue [21] did not activate the cytochrome c_1 promoter.

To help identify the cytochrome c_1 E2F1 activation region, SL2 cells were transfected with 5'- and 3'-deletion constructs (Figure 2). Transfection of 5'-deletion constructs (Figure 2, upper panel) discloses an E2F1-dependent activation site between nt -35 and -13. However, transfection of 3'-deletion constructs (Figure 2, lower panel) discloses a second E2F activation site between nt $+1$ and $+29$. Removal of either site, at nt -35 to -13 or nt $+1$ to $+29$, eliminates E2F1 activation, suggesting that the sites function co-operatively. Together, the two E2F1 activation sites cover a region between nt -35 and $+29$. This region also contains a third E2F-like site ($-4/+4$) which was found by computer search [8], but does not appear to support E2F1 activation in transfection experiments.

As shown in Figure 2, activation by E2F1 is neither additive nor co-operative with Sp1. This conclusion is strengthened by the observation that removal of nt -35 to -13 (containing both E2F and Sp1 activating sites) eliminates activation by both E2F1 and Sp1, whereas removal of nt $+1$ to $+29$ (containing an E2F protected site, see below), affects only the activation by E2F1.

The above experiments suggest that E2F1 activation of the cytochrome c_1 promoter involves two binding sites, one on either side of the transcription start site. The presence of these sites was confirmed by DNase I protection of the cytochrome c_1 proximal promoter region (nt $-72/+128$) using purified recombinant human GST-E2F1 protein (Figure 3). Two strongly protected regions, between nt -25 and -8 and between nt $+13$ and $+30$, were observed, which agree precisely with the location of the E2F activation sites determined by transfection experiments. The $-25/-8$ protected region includes the C-box Sp1-binding site, which is part of the Sp1 activation site [14]. The E2F-like
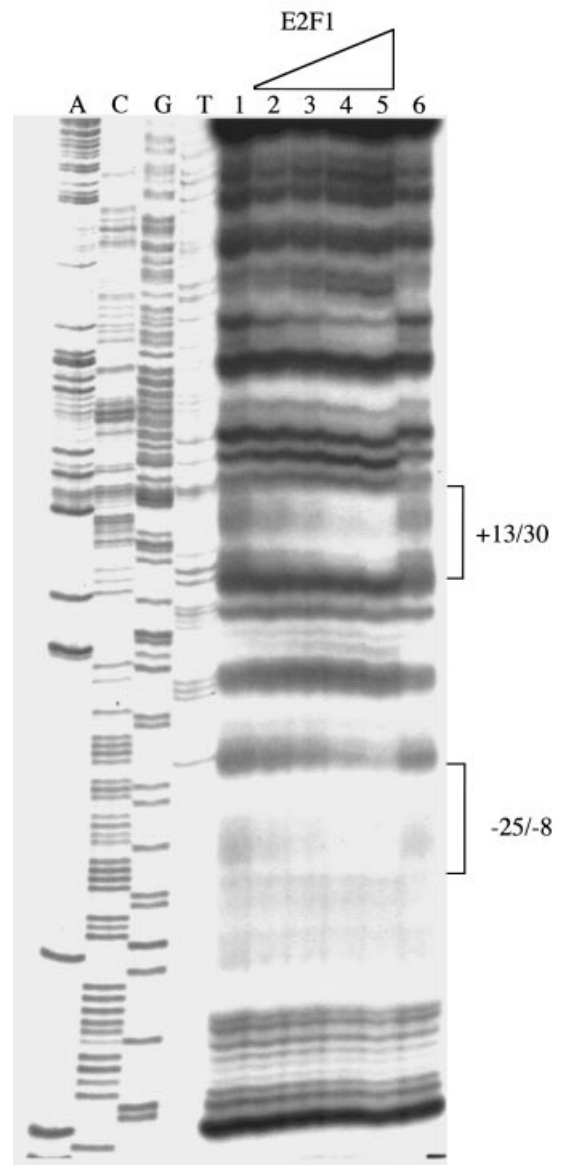

Figure 3   Identification of E2F1-binding sites in the cytochrome c_1 promoter

DNase I protection of the cytochrome c_1 ($-72/+128$) promoter fragment was performed with purified human GST-E2F1 (0.125, 0.25, 0.5 and 1 footprinting units). Lanes 1 and 6 contain no protein. The sequence for the coding strand is shown. Nucleotide numbering is relative to the transcription start site.

sequence ($-4/+4$) identified by computer search was not protected by E2F1, which also agrees with the results of our transfection experiments. Thus E2F1 binds most strongly to two 17-bp sequences that are separated by a 20-bp spacer.

The downstream cytochrome c_1 half site ($+13/+30$) appears to be recognized by the DNA-binding domain of E2F1. This is demonstrated in Figure 4(A) where E2F1 binding to the cytochrome c_1 ($+6/+30$) probe is inhibited by an oligonucleotide bearing the consensus E2F1 element, but not by an oligonucleotide bearing a mutated E2F element. Furthermore, E2F1 binding to the $+6/+30$ probe appears to be limited to nt $+12$ to $+30$ since a partially overlapping oligonucleotide (nt $-13/+12$) did not compete (Figure 4B). This result exactly confirms the E2F1-binding site ($+13/+30$) determined by DNase I
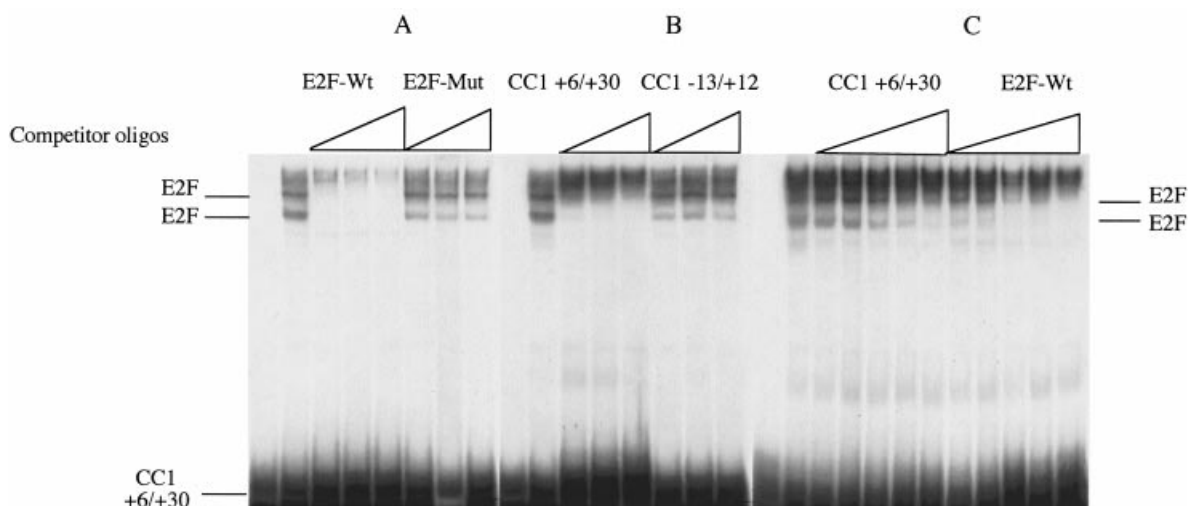
Figure 4   E2F1 binds to the cytochrome c_1 $+13/+30$ region in a specific manner

The gel mobility-shift assay was performed with purified, recombinant GST-E2F1 protein and an oligonucleotide probe (CC1 $+6/+30$) that covers the E2F1 protected region (nt $+13$ to $+30$) of the cytochrome c_1 promoter. (**A**) Competitor oligonucleotide E2F-Wt, containing a consensus E2F-binding element (TTCGCGC), and oligonucleotide E2F-Mut (TTCGATC) were added to 25-, 50- and 100-fold molar excess. (**B**) Competitor oligonucleotides CC1 ($+6/+30$) and CC1 ($-13/+12$) from the cytochrome c_1 promoter (see Figure 1) were added to 25-, 50- and 100-fold molar excess. (**C**) Competitor oligonucleotides were added to 1-, 2-, 5-, 10- and 25-fold molar excess.
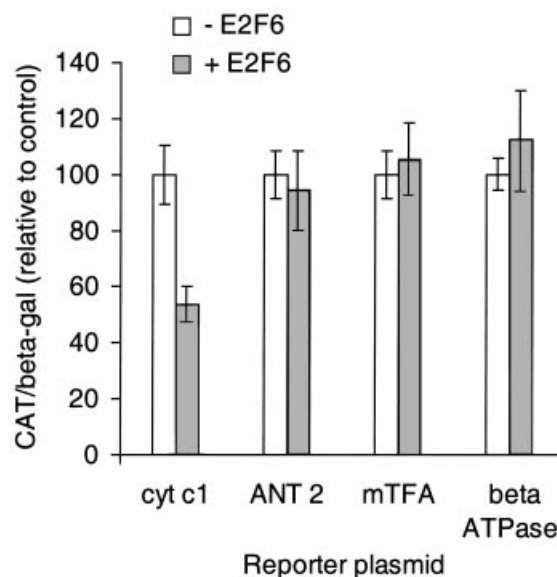
Table 1   E2F1 transcription activation is specific for the human cytochrome c_1 promoter

SL2 cells were transfected with reporter plasmids containing the promoters of four human mitochondrial genes. Reporter plasmids were transfected with individual expression vectors (pPac-E2F1, pPac-Sp1) or a combination of both as described in the Experimental section. Activation was calculated as fold activation relative to the expression of individual pCAT constructs in the absence of mammalian transcription factors. The values are given as the means $\pm$ S.D. from triplicate samples.

		Activation (fold induction)		
Promoter	Activation by …	E2F1	Sp1	E2F1 + Sp1
Cytochrome c_1 ($-1339/+128$)		61.6 ± 23.6	72.2 ± 29	56.8 ± 27.6
ANT2 ($-1237/+46$)		4.1 ± 2.6	45.7 ± 9.5	50.2 ± 13.9
F_1-ATPase β-subunit ($-593/+206$)		1.8 ± 0.4	20.6 ± 8.5	17.9 ± 2.2
mtTFA ($-643/+92$)		2.2 ± 0.7	24.4 ± 3.6	20.4 ± 2.7

Figure 5   E2F6 selectively inhibits the cytochrome c_1 promoter in transfected HeLa cells

Hela cells were transfected with reporter genes driven from promoter regions of the cytochrome c_1 (cyt c1), mtTFA, F1-ATPase β-subunit (beta ATPase) or the ANT2 genes, either alone or together with pCDNA3-HA-E2F6 that expresses human E2F6. The data are the means $\pm$ S.D. for three independent experiments in which all data points were collected in triplicate.

protection. The E2F1-footprinted site at $+13/+30$ contains a putative E2F element (TGGCGCCC) that differs from the consensus E2F element (TCGCGCCC) by a single nucleotide. Even so, the relative affinity of E2F1 for the cytochrome c_1 oligonucleotide containing the $+6/+30$ element appears from PhosphoImager analysis of the electrophoretic-mobility-shift-assay competiton experiments to be approx. 10-fold lower than for the E2F consensus element (Figure 4C).

To test whether E2F1 activates other OXPHOS gene promoters, we compared the promoter regions of three additional human mitochondrial genes; ANT2 ($-1237/+46$), the F_1-ATPase β-subunit ($-593/+206$) and mtTFA ($-643/+92$). Although all of the OXPHOS promoters tested were activated by Sp1 alone, only the cytochrome c_1 promoter was activated by E2F1 (Table 1). These data, together with DNase I footprint data showing an interaction of E2F1 only on the cytochrome c_1 promoter (results not shown), and data from HeLa cells (see below), suggest that E2F1 activates the cytochrome c_1 promoter relatively specifically.

To determine if E2F modulates cytochrome c_1 promoter activity in mammalian cells, we used a transcription-repression assay based on co-expression of E2F6. E2F6 lacks the trans-activation domains of E2F1, but contains the DNA-binding domains and specific, unique repressor domains [17,22–24]. Cytochrome c_1 promoter activity was suppressed by approx. 50 % in cells expressing E2F6 (Figure 5). By contrast, E2F6

expression had no effect on the ANT2, F_1-ATPase β-subunit or mtTFA promoters.

DISCUSSION

The human cytochrome c_1 promoter can be modulated by both E2F1 and E2F6. E2F1 is a strong activator in *Drosophila* cells, whereas E2F6 partially represses promoter activity in HeLa cells. These effects of the E2F proteins were not found in three additional promoters from OXPHOS genes, suggesting that E2F proteins are not general modulators of OXPHOS gene expression. Although the effects of E2F1 are specific for the cytochrome c_1 promoter, the detailed mechanism by which this promoter is modulated, and its physiological role in regulating cytochrome c_1 expression, remain to be elucidated.

The exact roles played by individual members of the E2F family in gene expression are still not clear. Although all members of the family are able to bind a consensus element, binding to DNA *in vivo* and functional responses appear to be influenced by promoter-element composition [22,23,25] and/or promoter context [26–30]. Thus it remains unclear which E2F family member(s) modulates the cytochrome c_1 promoter *in vivo*, even though we show activation by E2F1 in the SL2 test system. Furthermore, since the E2F proteins function both as activators and suppressors (see [31,32] for review), we cannot safely predict the nature of the modulation exerted on the cytochrome c_1 promoter *in vivo*. For example, our finding that E2F6 inhibits promoter activity in HeLa cells could be due either to active repression by E2F6 through a repression domain on the protein [22], or to prevention of activation by other E2F proteins as a result of competition for the E2F-binding site. The results do confirm, however, an involvement of E2F in modulating the cytochrome c_1 promoter in mammalian cells.

Although the exact role played by E2F in cytochrome c_1 expression remains to be elucidated, there is support for the notion that E2F proteins can repress expression of mitochondrial genes. Rohde and colleagues [33] showed that several mitochondrial proteins were up-regulated by overexpressing retinoblastoma (Rb) protein in Rb-deficient cells. Since expressed Rb is expected to bind E2F1, these data are consistent with a model in which free E2F present in Rb-deficient cells represses mitochondrial gene expression. Repression of cytochrome c_1 by E2F proteins would also be consistent with the observation that E2F sites located within the -50 to $+20$ region of TATA-less promoters function predominantly as repressor elements [30].

Finally, the significance of three potential E2F-binding sites in the cytochrome c_1 promoter (see Figure 1) remains to be studied. Our data from SL2 cells showing an apparent co-operativity between sites on either side of the transcription start site might not, as discussed above, reflect their usage *in vivo*. Both sites contain well-conserved E2F consensus sequences; TGGCGCC in the downstream site and an inverted TCGCGCC element in the upstream site. The latter comprises the 3′-half site of an inverted repeat footprinted by E2F1 (Figure 1). A TGGCGCC element is also present in the hamster ovary cell DHFR promoter, where it is bound by a distinct E2F species throughout the cell cycle. In contrast, the TCGCGCC element on the same promoter binds a second E2F species, but only during the G_1-to-S-phase transition [27]. The authors suggest that E2F binding to the TGGCGCC element might participate in basal transcription. A similar function for E2F on the cytochrome c_1 promoter is appealing, since, to date, the only physiological condition known to alter cytochrome c_1 expression is thyroid status [34,35], and this is most likely to be a permissive, rather than regulatory, effect.

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