Activating E2Fs mediate transcriptional regulation of human E2F6 repressor

Tarrah E. Lyons, Maysoon Salih, and Balwant S. Tuana

Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

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Lyons, Tarrah E., Maysoon Salih, and Balwant S. Tuana. Activating E2Fs mediate transcriptional regulation of human E2F6 repressor. Am J Physiol Cell Physiol 290: C189–C199, 2006. First published August 17, 2005; doi:10.1152/ajpcell.00630.2004.—E2F6 is believed to repress E2F-responsive genes and therefore serve a role in cell cycle regulation. Analysis of the human E2F6 promoter region revealed the presence of two putative E2F binding sites, both of which were found to be functionally critical because deletion or mutations of these sites abolished promoter activity. Ectopic expression of E2F1 protein was found to increase E2F6 mRNA levels and significantly upregulate E2F6 promoter activity. Deletion or mutation of the putative E2F binding sites nullified the effects of E2F1 on the E2F6 promoter activity. Studies on the temporal induction of E2F family members demonstrated that the activating E2Fs, and most notably E2F1, were upregulated before E2F6 during cell cycle progression at the G1/S phase, and this coincided with the time course of induction experienced by the E2F6 promoter during the course of the cell cycle. EMSAs indicated the specific binding of nuclear complexes to the E2F6 promoter that contained E2F1-related species whose binding was specifically competed by the consensus E2F binding site. Chromatin immunoprecipitation assays with anti-E2F5s demonstrated the association of E2F family members with the E2F6 promoter in vivo. These data indicate that the expression of the E2F6 repressor is influenced at the transcriptional level by E2F family members and suggest that interplay among these transcriptional regulators, especially E2F1, may be critical for cell cycle regulation.

Smyth Rd., Ottawa, ON, Canada K1H 8M5 (e-mail: btuana@uottawa.ca).

SEVERAL GROUPS OF TRANSCRIPTION factors function to impose controls over cell cycle entry, progression, and exit (2, 14, 25). A prominent family of cell cycle regulators is the E2Fs, which are DNA-binding heterodimeric transcription factors that recognize the consensus sequence TTTSSCGC in the promoters of their target genes to regulate their expression (8, 19, 28). The E2Fs control the expression of many genes, particularly those that act in the transition from G1 to S phase of the cell cycle (12, 14, 28). These genes include cyclins E and A, c-myc, those that act in the transition from G1 to S phase of the cell cycle; transcriptional control

E2F6 is a weak transactivator that attenuates the transcription of E2F-responsive genes to regulate cell cycle exit and terminal differentiation (25). E2Fs 6 and 7 are the most recently discovered members of the E2F family and are believed to repress E2F-responsive genes (1, 3, 4, 6, 26).

The biological activity of E2F6 is not fully understood, although current studies support a unique role for E2F6 as a transcriptional repressor. Recent studies have defined the structure of the human and mouse E2F6 gene, which is composed of eight exons and is subject to alternative splicing that may give rise to as many as three distinct E2F6 gene products (3, 10, 11, 21, 22). The unique structural feature of E2F6 compared with the other E2Fs is that it lacks the carboxy-terminal domains for pocket protein binding and transactivation (6, 26). E2F6 has instead a repression domain shown to associate with members of the mammalian polycomb complex, and it is believed that via this interaction, it binds to promoter regions to repress E2F-responsive genes (27). It has been shown that E2F6 overexpression can exert an inhibitory effect on S phase entry and can induce subsequent proliferative arrest (S phase accumulation) in NIH 3T3 cells (1, 6). To elaborate further on the biological role of E2F6, we have followed up the cloning of the human and mouse E2F6 promoters (11, 12, 21, 22) with an investigation of the endogenous expression profile of the gene, as well as its transcriptional regulation during the cell cycle. The data reported here show that the human E2F6 gene is regulated in a cell cycle-specific manner and that its transcriptional control may be mediated to a significant extent by E2F1.

EXPERIMENTAL PROCEDURES

Isolation and characterization of human genomic clones. One million plaque-forming units of Lambda FIXII Human Genomic Library (Stratagene no. 946205) were plated according to the manufacturer’s recommendations. Colony/plaque screen hybridization transfer membranes (Dupont) were probed with a 75-bp oligonucleotide containing 32P-labeled dCTP with the entire alternative exon of human E2F6. It was generated by PCR using the primers 5′–GACGGAGGAGCGGTTCGCG–3′ and 5′–CCGGCTTCCGCGATAGAAC–3′. The PCR product was then digested with BsrXI, which removed 44 bp of the nonalternative exon sequence to distinguish the spliced gene from the unspliced E2F6 present on chromosome 22. The hybridization was performed at 65°C (in 10% polyethylene glycol, 1.5% sodium chloride-sodium phosphate-EDTA, and 7% SDS) for at least 12 h, followed by four washes in 0.2–1× SSC, 0.1% SDS at 50°C. The membranes were then exposed to Biomax MR films (Amersham). A positive phage clone was identified on tertiary screening and DNA was prepared as previously described (23), using XL1-Blue MRA (P2) as a host. DNA was digested with different cell cycle; transcriptional control
restriction enzymes and analyzed by Southern blot hybridization with the same probe used to screen the library and other sequences from different regions of the gene. Positive fragments were subcloned into the appropriately digested pBluescript SK and subsequently sequenced. Human genomic DNA was analyzed for restriction sites in the region of the E2F6 promoter. Several sequences were excised from the E2F6 clones with restriction enzymes and cloned upstream of the luciferase reporter gene in the pGL3-Basic vector. The minimal promoter was determined by transiently transfecting MCF-7 cells with each of the promoter constructs and measuring relative luciferase activity [normalized to β-galactosidase (β-gal) activity] of whole cell extracts. 5’-Rapid amplification of cDNA ends (5’-RACE) was carried out using GeneRacer (Invitrogen) with mRNA isolated from cells. mRNA was decapped and ligated to the kit oligo. mRNA was reverse transcribed to provide cDNA for the 5’-RACE. Amplification was then performed with a gene-specific primer and a primer complementary to the kit oligo. Purification, cloning, and sequencing revealed the location of the transcription start site.

**Cell lines and cell culture.** HEK-293 cells were grown in DMEM (GIBCO) at pH 7.28 with 10% fetal bovine serum and 0.1% gentamicin-reagent solution (GIBCO). MCF-7 cells were grown in DMEM at pH 7.28 with 5% fetal bovine serum, 0.1% gentamicin-reagent (GIBCO), and 1% MEM nonessential amino acid solution (GIBCO). Cells were maintained at 37°C and 5% CO2.

**Transient transfection.** Cells were seeded to a density of 1.0 × 10⁶ in 100-mm plates or to 3.0 × 10⁵ in 60-mm plates. During the exponential growth phase 24 h after seeding, cells were transfected with plasmid DNA using Fugene transfection reagent (Roche) according to the manufacturer’s recommendations.

**Generation of stable clones.** The cytomegalovirus (CMV) promoter was excised from pcDNA3 (5.4 kb) with NrdI and EcoRV (Invitrogen). An E2F6 promoter-luciferase fusion was removed from an existing pGL3-Basic vector with ClaI and XbaI (Invitrogen) and blunted with Klenow fragment (Invitrogen). Ligation between the pcDNA3-CMV and the luciferase-promoter fragment yielded the desired fusion construct containing resistance to G418 (neomycin). DH5α cells were transformed with the ligation and plated on agar plates with ampicillin at 37°C. Plasmids were isolated from selected colonies and checked for correct insertion orientation. Cells were transiently transfected with the luciferase fusion construct in a 100-mm plate as described above. Twenty-four hours after transfection, cells growing at near confluence were treated with 100 mg/ml G418 to a final concentration of 2.5 μg/ml for 8–10 days. Visible colonies were selected and grown in media containing G418 at 2.5 μg/ml. Cell samples from each plate were tested for luciferase expression (17).

**Plated cell synchronisation for time course.** Adherent cells were treated with 1 mg/ml nocodazole (Sigma) to a final concentration of 250 ng/ml (6) to impose a cell cycle block at G2/M. Cells were incubated for 16–20 h at 37°C and then released with fresh medium.

**Reporter gene assays.** Luciferase assays were performed with a luciferase reporter gene assay kit (Roche no. 1897667), and β-gal assays (16) were performed with a β-gal kit (Stratagene no. 200383).

**Western and Northern blots.** Whole cell extract was prepared with 1× cell lysis buffer (Roche no. 1897667) in sterile double-distilled water. Buffer was treated with the protease inhibitor Complete Mini (Roche) at one tablet per 10 ml of buffer. The protein concentration of the whole cell lysates obtained from each time course sample was measured by the bicinchoninate protein assay method (Pierce) according to the manufacturer’s recommendations. Twenty micrograms of each protein-containing sample were subjected to SDS-PAGE. The gel was transferred to a positively charged nitrocellulose membrane and probed with an appropriate primary antibody [anti-E2F1 to anti-E2F6 (Santa Cruz) and anti-GAPDH (Advanced Immunochemical)] followed by the corresponding horseradish peroxidase-conjugated secondary antibody.

RNA was isolated from cells with Tripure isolation reagent (Roche) according to the manufacturer’s instructions. Ten micrograms of each RNA sample were added to 15 μl of RNA loading buffer (900 μl of deionized formamide, 180 μl each of formaldehyde and 10× MOPS buffer, 168 μl of gel loading buffer, and 20 μl of ethidium bromide). Samples were denatured at 65°C for 15 min and were loaded on a prerun 1.0% agarose gel (6% formaldehyde and 10× MOPS buffer). The gel was transferred through capillary action to a nylon membrane overnight. It was probed with a 0.9-kb PCR-generated cDNA probe with incorporated digoxigenin (Dig)-labeled UTP nucleotides, using the primers 5’-TCTGGCTTCGTGGCTAG-GCT-3’ and 5’-CGCTACTGAGAACGAGAGCACGCAC-3’.

**Flow cytometry.** Cells in 60-mm plates were trypsinized and treated with 1× PBS-EDTA. Samples were spun down, and pellets were resuspended and fixed in 1 part 1× PBS-EDTA and 3 parts 70% ethanol and then stored at −20°C. At the time of analysis, cells were washed and resuspended in PBS-EDTA. Cells were treated with RNase at a 1:50 dilution and stained with propidium iodide at 1:500 before cell cycle analysis on a flow cytometer at the Ottawa Regional Cancer Center (Ottawa, ON, Canada).

**Site-directed mutagenesis.** Complementary primers 5’-CAGTGGTTCGACATTCGCTTCGCTC-3’ and 5’-GAGGCAACCGTGCTTGGACCGCGG-3’ were designed with a mutated putative E2F1 binding site (GAGCACGG). With the use of the QuickChange mutagenesis kit (Stratagene no. 200519) according to the manufacturer’s instructions, PCR amplification of the template (E2F6 promoter-luciferase construct i: −597 to +96) incorporated the mutation. Supercompetent bacterial cells were transformed with the product, and the resultant colonies were picked, cultured, and used to generate miniprep DNA for sequencing.

**Site deletion by PCR amplification.** PCR amplification with primers 5’-CTCAGCTGTGACCCGTACCCG-3’ and 5’-TACACGGCGCCC-CTCTGTGCGGTATG-3’ yielded a fragment from −335 to +13 of the transcriptional start site within the E2F6 promoter that eliminated the putative E2F1 binding site at +24. The product was cloned into PGL3-Basic upstream of the luciferase reporter gene.

**EMSA and supershift.** EMSAs involving chemiluminescence-based detection were carried out with the LightShift Mobility/Shift Assay kit (Pierce no. 20148) according to the manufacturer’s instructions.

Nuclear extracts were taken from MCF-7 cells overexpressing E2F1, using the N-Per nuclear and cytoplasmic extraction reagents (Pierce no. 78833). Sixty-base pair sense and antisense oligonucleotides containing the putative E2F1 binding sites were 3′ end labeled with a biotin 3′ end labeling kit (Pierce no. 89818). The oligo upper strands used were as follows for the human putative E2F1 sites at −174 and +24, respectively: 5’-CACCCTGCAGATAAGCTCGTGGTTTTCCCCGTTGCTCTTCTGACCCCG-3’ and 5’-TG-CGGCAAGGGGCGGGTGACTCGCGCATGCGGAAGATGGCG-GGCGGCGGACTTGGAT-3’.

Nuclear extracts were incubated with the probe in a 25-μl sample and loaded onto a 16 × 18 × 0.1-cm 5% native polyacrylamide gel. Protein samples were also incubated with an excess of unlabeled target DNA to compete labeled probe binding. For supershifts, nuclear extract was treated with 1–2 μl of E2F1 antibody (anti-E2F1sc-I93X polyclonal; Santa Cruz) for 1 h at room temperature before incubation with the probe. For the chemiluminescence-based assays, the membrane was transferred electrophoretically to a BioDyne-B nylon membrane (Pierce no. 77016), and chemiluminescence detection was carried out according to the manufacturer’s instructions.

EMSAs involving radiolabeled oligonucleotide probes were carried out as follows. Nuclear extracts were taken from HEK-293 cells overexpressing E2F1 with the N-Per nuclear and cytoplasmic extraction reagents (Pierce no. 78833). Twenty-four-base pair complementary DNA strands (5’-GTACCTGGCCATCGGGGAAGTGGC-3’) containing the putative E2F1 binding site GCGGGGAG were annealed and radiolabeled with [γ-32P]ATP, using T4 polynucleotide kinase. Nuclear extracts were incubated with the probe in a 30-μl
sample and loaded onto a 16 × 18 × 0.1-cm 5% native polyacrylamide gel. Protein samples were also incubated with an excess of unlabeled target DNA to compete labeled probe binding. The gel was wrapped and exposed to autoradiography film overnight.

**E2F1 overexpression.** A plasmid containing the complete cDNA of the human E2F1 gene was transiently transfected into cells that carried the E2F6 promoter-luciferase reporter with Fugene transfection reagent (Roche) according to the manufacturer’s recommendations. Control cells were transfected with only the promoter-reporter gene construct.

**Chromatin immunoprecipitation.** In vivo detection of E2F6 promoter-associated E2Fs was performed by chromatin immunoprecipitation (ChIP) (13). Human HEK-293 cells were treated with 1% formaldehyde to fix protein-DNA complexes. Isolated chromatin was sonicated to lengths between 1.0 and 2.0 kb and was precleared with salmon sperm DNA to reduce nonspecific background. DNA was immunoprecipitated overnight with antibodies against E2F1 (sc-193x, Santa Cruz), E2F2 (sc-633x, Santa Cruz), E2F3 (sc-897x, Santa Cruz), E2F4 (sc-866x, Santa Cruz), and E2F5 (sc-999x, Santa Cruz). Controls included PCR reactions using a control template (no E2F6 promoter) and DNA immunoprecipitated with either IgG or no antibody. DNA-protein-antibody complexes were recovered on agarose beads and washed. The cross-links to each of the antibody-protein complexes were reversed on DNA eluted from the beads, which was subsequently used as a template in PCR with E2F6 promoter-specific primers (5′-GAGCTCAGGAAATAGGAG-3′ and 5′-ATACTGAAATTTCTGCTGAGT-3′) or SLMAP promoter-specific primers (5′-CTGACTCGAGGTTGGCCCATGTGCTGAC-3′ and 5′-GACTCGCCGACACAGGAAATGCA-3′).

**RT-PCR.** Total RNA was extracted from MCF-7 cells transfected with an empty pcDNA3 vector (control) and MCF-7 cells transfected with an E2F1 expression vector. The RNA was subjected to an RT-PCR reaction with the One Step RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. A master mix was created for the PCR reactions involving buffer, water, and template, and the mix was divided among all the reactions before addition of primers specific to each reaction or enzyme mix. This was to ensure a consistent amount of template in each reaction. For the test reactions targeting the E2F6 mRNA, forward and reverse primers specific to the E2F6 cDNA were used as follows: 5′-GGTTGCAAGAAACTGGGAG-3′ and 5′-CGCTACTGAGGAGGAGCTG-3′. For the control reactions targeting GAPDH mRNA, the same template was used in RT-PCR with primers as follows: 5′-GCAGAACATTCTGAGGGAGGAGATGC-3′ and 5′-CTCCGGGCTCTTCTGGCT-3′.

**Software.** MatInspector (Genomatix Software 1998–2004) online software, which utilizes a library of matrix descriptions for transcription factor binding sites to locate matches in submitted sequences, was used.

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**Fig. 1.** The human and murine E2F6 promoters. Shown are the promoter sequences for the human and mouse E2F6 genes that were cloned upstream of luciferase for the purpose of promoter analysis in this study. A segment spanning −571 to +104 of the human promoter was used, as well as a segment spanning −205 to +273 of the murine promoter. Index at left refers to the transcription start site indicated at +1 for both the human and mouse promoters, based on the largest protected product from RNase protection analysis (4, 12). Also shown are known and putative consensus sequences for the transcription factor binding sites to locate matches in submitted sequences, was used.
RESULTS

Putative E2F binding sites in human E2F6 promoter. To assess the regulation of the E2F6 gene, we isolated the human E2F6 promoter (Refs. 21 and 22; Salih and Tuana, GenBank accession no. AY083997, 2002), and this was identical to that reported by Kherrouche et al. (11). Sequence analysis of the human E2F6 promoter (Fig. 1) indicated the presence of two putative E2F1 binding sites; GCGGGAAG was at +24 downstream from the translational start site (+1), and the second site (TTCTCCGG) was at −174 upstream of the translation start site (MatInspector). In comparison, study of the murine promoter predicted the presence of one E2F consensus site at +15 downstream of the translational start site (10). It is the structural and positional similarity between the E2F binding site at +15 in the mouse promoter and the putative E2F binding site at +24 in the human promoter that prompted interest in this site as a potential functional E2F consensus sequence. To develop an understanding of the importance of these sites to E2F6 promoter activity, deletions of the promoter region were cloned in frame with the reporter gene luciferase. The relative induction of the cloned regions of the promoter and restriction digests of the promoter showed variation in activity likely due to the exclusion and/or inclusion of putative upstream and downstream binding sites that exist for transcriptional regulators, which include the putative E2F consensus binding sites at +24 and −174.

Transcriptional regulation of human E2F6 gene during cell cycle. We sought to determine whether E2F6 is transcriptionally regulated in a cell cycle-dependent manner. The promoter-luciferase gene fusion construct i encompassed the largest promoter region and was used to determine the E2F6 promoter induction profile over the course of the cell cycle (Fig. 3). Stably transfected HEK-293 cells were synchronized at the G2/M boundary with nocodazole as validated by flow cytometry (Fig. 3D). Whole cell lysates were analyzed over 12 h for luciferase activity, which was expressed as a relative change in induction from that observed at time 0 (G2/M). The data indicate an approximate threefold increase in luciferase activity at the 6-h postsynchronization time point (Fig. 3A), which, according to flow cytometry results (Fig. 3D), corresponds to the G1/S phase transition in HEK-293 cells. To examine fluctuation in E2F6 message levels, RNA was extracted from G2/M-blocked HEK-293 cells and analyzed in Northern blots with a Dig-labeled E2F6 cDNA probe (Fig. 3B). The mRNA expression profile consistently indicated two E2F6 transcripts of 3.9 and 2.5 kb, which also peaked at the 6-h time point, consistent with previous data (1, 6, 26). To examine changes in endogenous E2F protein levels during the cell cycle, cell lysate was resolved by SDS-PAGE and subjected to Western blot analysis with anti-E2F6 (Fig. 3C). The data show that a
Apolipoprotein 34 kDa and a doublet of ~32 and ~31 kDa were specifically recognized by anti-E2F6 and expressed in a cell cycle-regulated manner, as reported previously (6, 26). The results show an increased intensity in both E2F6 mRNA and protein expression at the 6-h time point after synchronization, which coincided with the increased promoter induction during the luciferase assay at this time (G1/S). Furthermore, the Western blot (Fig. 3C) shows that the peak temporal expression of E2Fs 1–3, and most apparently E2F1, seems to manifest at 5 h after synchronization, whereas the expression levels of E2Fs 4 and 5 remain relatively unaltered during cell cycle progression. These data suggest that the activating E2Fs are induced before the induction of E2F6 and may serve a role in E2F6 activation.

**E2F1 regulates E2F6 mRNA expression and binds putative E2F binding sites in E2F6 promoter.** Because the E2F6 expression profile follows that of the activating E2Fs during the
cell cycle, we sought to determine whether increased levels of E2F 1 could influence E2F6 mRNA levels. E2F1 expression plasmid was transiently transfected into MCF-7 cells, and semiquantitative RT-PCR was performed on RNA extracted from these cells to determine the effects on the level of E2F6 mRNA expression (Fig. 4). The E2F6 transcripts were elevated ~1.3-fold (determined by densitometry) in the E2F1-transfected cells at 48 h after transfection compared with empty pcDNA3 vector-transfected cells. A Western blot was performed on total cell extract from the transfected cells, using antibodies against E2F1 and E2F6 to verify overexpression of E2F1 and its effects on E2F6 protein levels. There was an ~1.3-fold increase in E2F6 protein expression due to E2F1 compared with GAPDH (Fig. 4C).

Because the human and mouse promoters contain putative E2F1 binding sequences, we sought to determine a molecular interaction between E2F1 and the E2F6 promoter that could account for the effects on gene expression. EMSAs were carried out with a radiolabeled 25-bp commercial oligo encompassing the known consensus sequence for E2F1 (TCTC-GGC) and a radiolabeled, synthesized 24-bp oligo containing the putative E2F binding site at +24 (GCGGGGAAG) shows a retardation of the probe on the gel relative to the lane containing only the probe (lane 2, Fig. 5A). The addition of an excess of unlabeled consensus probe caused the complex to be competed out (lane 6, Fig. 5A), as shown for the consensus sequence (lane 3, Fig. 5A).

When a probe containing the second putative E2F1 binding site at −174 was used in an EMSA, the shift was similar to that observed with the probe containing the binding site at +24 (Fig. 5A, lane 5, and Fig. 5C, lane 2). This band shift was similar to that observed with the probe containing the binding site at +24 (Fig. 5A, lane 5, and Fig. 5C, lane 2). The addition of an excess of unlabeled probe caused the complex to be competed out (Fig. 5A, lane 6).

To test whether the protein-DNA complexes in the EMSAs contained E2Fs, a polyclonal antibodies against the E2Fs were used in supershift assays involving the two putative E2F sites in the human E2F6 promoter at +24 (Fig. 5B) and −174 (Fig. 5C). The data in Fig. 4, B and C, show diminished intensity at the level of the band shift with anti-E2F1 for the site at +24 and −174, with the appearance of a higher-molecular-weight complex, indicating that some of the protein-DNA complex was successfully recognized by the E2F1 antibody. On the basis of the comparable positions of the band shifts and supershifts, both putative E2F1 binding sites in the human E2F6 promoter were recognized by a nuclear protein complex that was immunochemically identified as containing E2F1. None of the other anti-E2Fs resulted in the protein-DNA complexes being retarded, as seen with E2F1 (data not shown).

To determine whether the putative E2F binding site (+15) in the murine promoter can bind nuclear factors as shown in the studies of the human promoter above, we performed an EMSA on mouse p19 cell nuclear extract (Fig. 5D). There is retardation of the probe in lane 2 relative to the lane containing the probe alone (lane 1) (Fig. 5D). This complex is specifically competed out by the addition of an excess of unlabeled probe (Fig. 5D, lane 3); however, it is retained when a cold probe containing a mutated version of the E2F consensus binding site is used in competition (Fig. 5D, lane 4).

**E2F targets E2F6 promoter in vivo.** Because EMSA results suggest that the E2F6 promoter contains sequences recognized by E2F1, we sought to determine the molecular basis for this interaction between E2F1 and the E2F6 promoter that could account for the effects on gene expression.
interaction in vivo. ChIP assay was used to determine whether there existed in vivo binding of E2F species to the E2F6 promoter in HEK-293 cells. Sonicated chromatin from a population of asynchronous cells was immunoprecipitated as a chromatin-protein complex by polyclonal antibodies raised against E2Fs 1–5. Primers specific to the E2F6 promoter (Fig. 6A) and to the control promoter SLMAP (Fig. 6B) were used in PCR amplification of a sample from each immunoprecipitation. PCR was also performed on a control template (not containing the E2F6 promoter), DNA immunoprecipitated with either IgG or no antibody, as well as the total input sample of chromatin attained before the immunoprecipitation step. PCR with the E2F6 primer set yielded a 372-bp product from the immunoprecipitations with anti-E2Fs 1–5, and this was sequenced and positively identified as a region of the E2F6 promoter spanning sequences −597 to −226 from the transcription start site. As expected, PCR with the SLMAP primer set yielded no product, because the SLMAP promoter has no consensus E2F binding sites (Salih M and Tuana BS, unpublished results).

![Graph A](http://ajpcell.physiology.org/)  
**A**  
Native Promoter  
+ E2F1

![Graph B](http://ajpcell.physiology.org/)  
**B**  
Native Promoter  
E2F1 site (a) deleted  
E2F1 site (a) deleted  
+ E2F1

![Graph C](http://ajpcell.physiology.org/)  
**C**  
Native Promoter  
E2F1 site (b) mutated  
E2F1 site (b) mutated  
+ E2F1

![Graph D](http://ajpcell.physiology.org/)  
**D**  
Control  
Native promoter  
Native + E2F1  
Deletion (+24)  
Mutation (-174)

![Graph E](http://ajpcell.physiology.org/)  
**E**  
Native promoter  
Mutant promoter

![Graph F](http://ajpcell.physiology.org/)  
**F**  
Control  
Native promoter  
Native promoter + E2F1  
Mutant promoter

**Fig. 7.** E2F1 binding induces the human E2F6 promoter. The effect of E2F1 transactivation on promoter activity was evaluated in MCF-7 cells synchronized at G2/M in the presence and absence of E2F1 overexpression on a promoter-luciferase construct containing the native E2F6 promoter (A), deleted E2F1 binding site a (GCGGGAAG; B), and mutated E2F1 binding site b (TTTCCCGG; C). D: effect of E2F1 overexpression, site deletion at +24, and site mutation at −174 on the activity of the human E2F6 promoter in asynchronous HEK-293 cells. E: activity of the native murine E2F6 promoter and the mutated murine E2F6 promoter with a compromised E2F1 consensus site in asynchronous p19 cells. F: effect of E2F1 overexpression and site mutation at +28 on the activity of the murine E2F6 promoter in asynchronous p19 cells.
E2F1 binding mediates E2F6 promoter activity. Having established that E2Fs with transcriptional activating properties bind to the human E2F6 promoter, we wanted to examine whether ectopic expression of E2F1 can induce E2F6 promoter activity (Fig. 7A). E2F1 was found to significantly enhance E2F6 promoter activity, resulting in a relatively high constitutive luciferase expression throughout the observed time course. These data indicate that irrespective of existing cell cycle controls at the level of the promoter, enhanced E2F1 levels have a marked effect on the activation of the E2F6 promoter.

To gauge the relative importance of the putative E2F1 binding sites to the maximal activity of the promoter, each site was independently compromised and the effect on the promoter induction was examined. The E2F binding site \( a \) at +24 was deleted, and E2F site \( b \) at −174 was subjected to site-directed mutagenesis. When MCF-7 cells were transiently transfected with a luciferase construct containing one of the compromised E2F binding sites, E2F6 promoter activity was significantly reduced compared with control cells with intact E2F binding sites (Fig. 7, B and C). A similar decrease in promoter activity was observed in mouse p19 cells when the E2F site at +15 in the mouse E2F6 promoter was mutated (Fig. 7E). These data show that this E2F binding is crucial to the control of the E2F6 promoter by the E2Fs. Subsequently, we wanted to examine whether ectopic expression of E2F1 could induce an elevated activity in the promoters compromised for E2F binding. The reporter gene constructs containing either mutant or deleted E2F1 sites were transiently cotransfected into MCF-7 cells with E2F1 expression constructs. Figure 7, B and C, shows that increased levels of E2F1 protein in the cell had a negligible effect on the nullified promoter activity when either site was compromised. These data further imply that E2F1 interactions at its binding sites may be essential for E2F6 promoter function.

The effects of mutation and deletion of the E2F binding sites on human and murine E2F6 promoter activity were compared in an asynchronous population of cells under similar conditions. Figure 7D indicates a more than twofold increase in human promoter activity in the presence of ectopic E2F1. Deletion of the putative site at +24 resulted in a significant decrease in promoter activity, as was the case with the mutation of the site at −174. When asynchronous mouse p19 cells were subjected to similar experimental conditions (Fig. 7F), an increase in promoter activity comparable to that observed in human cells was observed in the presence of ectopic E2F1. The mutation of the E2F binding site at +15 abolished promoter activity. These results show that E2F1 is capable of enhancing the activity of both the human and the mouse E2F6 promoter, and the putative E2F binding sites at +24 and −174 for human and +15 for mouse may be viable transcription factor binding sites that are important to the regulation of the E2F6 gene.

**DISCUSSION**

The data presented herein show that the E2Fs, and most evidently E2F1, in part mediate the transcriptional control of the E2F6 gene. Functional analysis of the promoter demonstrated that transcriptional mechanisms drive the expression of the E2F6 gene in a cell cycle-specific manner. Structural analysis of the human E2F6 promoter revealed the presence of two putative E2F consensus binding sites where the E2Fs detected in the ChIP assay could potentially bind. The association of E2Fs with the human E2F6 promoter was further investigated with EMSAs that were used to determine whether the E2Fs were recognizing the putative E2F consensus sequences found at +24 and −174 relative to the transcription start site. The data show that these binding sites, as well as a putative site found at +15 in the mouse promoter predicted previously (10), were able to complex specifically with nuclear complexes containing E2F1. When these putative E2F binding sites were compromised by deletion or mutation, the activity of the human and murine promoters was significantly diminished.
Furthermore, E2F6 mRNA expression as well as E2F6 promoter activity were enhanced by the ectopic expression of E2F1. Interestingly, this enhancement in human promoter activity was found to be dependent on the integrity of the two E2F binding sites.

We found that the human E2F6 promoter was induced in a cell cycle-specific manner, with peak activity at G1/S. The temporal induction of E2Fs 1–3 that occurs just before the upregulation of E2F6, as demonstrated by their expression profiles, suggests that the transactivating E2Fs may be upstream regulators of E2F6 gene expression. Two mRNA transcripts and three proteins encoding E2F6 were consistently detected, with the upregulation noted at the G1/S phase of the cell cycle as defined previously (3, 6, 26). Thus these data collectively suggest that E2F1 may derive E2F6 gene expression at the level of the promoter in a cell cycle-dependent manner.

Our data presented herein imply the existence of a potential novel feedback mechanism through E2F1 transactivation of the repressor E2F6. Thus E2F6 would experience upregulation at G1/S transition to exert an opposing effect on the activities of E2F-responsive promoters. We propose a model in which E2F recognizes two consensus sequences on the human E2F6 promoter to play a primary role in its induction (Fig. 8). The cell cycle mediation imposed by E2F6 would be exerted by interfering in the transcriptional activation of E2F target genes. Because such genes are required for cell cycle progression and S phase entry, the repressive effects of E2F6 therefore direct the orderly progression of the cell cycle and ultimately appropriate cell cycle exit and differentiation (5, 7, 24, 28). The mechanism of repression by E2F6 is not yet clear, although recent studies have shown that E2F6 interacts with certain repressive factors that associate with the multimeric mammalian polycomb protein complex through its repression domain (26, 27). This suggests that the complex is required for the gene-silencing effect of E2F6, which is thought to occur through the recognition of consensus sequences because E2F6 does bind DP to form a functional DNA binding complex through its domains for heterodimerization and DNA binding (12, 19, 26, 27). In fact, ChIP assays have shown that E2F6 associates with and negatively regulates many loci that contain E2F1 binding sites, such as in the promoters of BRCA1 (tumor suppressor), TRFP (transcription mediator), NEK11 (DNA replication), and DHPS (amino acid synthesis) (18).

The data presented herein show that the cell cycle-specific expression of the E2F6 gene is mediated by transcriptional mechanisms that operate at the level of the promoter, where E2F1 may be a principal transcriptional regulator of the E2F6 repressor.

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REFERENCES


