Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter

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To prepare for the DNA synthesis (S) phase of the cell cycle, transcription of many genes required for nucleotide biosynthesis increases. The promoters of several of these genes contain binding sites for the E2F family of transcription factors, and, in many cases, mutation of these sites abolishes growth-regulated transcription. The RNA levels of one family member, E2F1, increase about 15-fold at the G1/S-phase boundary and expression of E2F1 in quiescent cells activates transcription from some G1/S-phase-specific promoters, suggesting that E2F1 plays a critical role in preparing cells to enter S phase. To elucidate the signal transduction pathway leading to the activation of genes required for DNA synthesis, we are investigating the mechanism by which expression of E2F1 is regulated. To determine whether levels of E2F1 mRNA are controlled by changes in promoter activity, we have cloned and characterized the mouse E2F1 promoter. Sequence analysis revealed two sets of overlapping E2F-binding sites located between −12 and −40 relative to the transcription initiation site. We show that these sites bind cellular E2F and that an E2F1 promoter fragment can be activated up to 100-fold by coexpression of E2F proteins. We also show that the activity of this E2F1 promoter fragment increases ~80-fold at the G1/S-phase boundary and that this activation is, in part, regulated by Go-specific repression via the E2F sites. However, the E2F sites are not sufficient to mediate growth-regulated transcriptional activity; our results indicate that multiple DNA elements are required for transcription regulation of the E2F1 promoter at the G1/S-phase boundary.

[Key Words: E2F; cell cycle; growth regulation; transcription; promoter]

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Cell growth is controlled by an orderly sequence of events termed the mitotic cell cycle. Each stage of the cycle is characterized by the expression of a set of genes required to progress through that stage. For example, to prepare for DNA synthesis in S phase, the levels of proteins required for nucleotide biosynthesis or utilization are increased in late G1. This increase is regulated by a transcriptional mechanism for dihydrofolate reductase (dhfr), thymidine kinase (tk), and DNA polymerase α [Farnham et al. 1993]. The promoters for each of these genes contain binding sites for the E2F family of transcription factors, and mutagenesis has demonstrated the critical role of these sites in regulation [Pearson et al. 1991; Means et al. 1992; Li et al. 1993; Dou et al. 1994]. The sequence of four different proteins that bind to an E2F consensus site [TTTSSCGC, S=C or G] has been reported; E2F1, E2F2, E2F3, and DP-1 [Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992; Girling et al. 1993; Ivey-Hoyle et al. 1993; Lees et al. 1993; Li et al. 1994]. E2F1, E2F2, and E2F3 are highly similar in their DNA-binding domains, whereas E2F1 and DP-1 are less closely related. Although E2F1 can bind to DNA as a homodimer, E2F1/DP-1 heterodimers have greatly increased DNA-binding activity and E2F1 and DP-1 can cooperate in trans-activation assays [Bandara et al. 1993; Helin et al. 1993; Ivey-Hoyle et al. 1993]. These data support a model in which one E2F and one DP subunit heterodimerize in cells to create functional E2F.

We have focused on understanding which of the E2F family members are involved in the regulation of specific G1/S-phase-activated genes that are required for DNA synthesis. We have shown that expression of E2F1 in serum-starved cells can activate some [DNA polymerase α, dhfr, and tk] G1/S-phase-regulated promoters [Li et al. 1994]. Also, E2F1 binding activity and mRNA levels fluctuate through the cell cycle, peaking at the G1/S-phase boundary [Kaelin et al. 1992; Chittenden et al. 1993; Slansky et al. 1993; Li et al. 1994; Shan et al. 1994; L.M. Bennett, P.J. Farnham, and N.R. Drinkwater, in prep] and expression of E2F1 can, in some cases, drive quiescent cells into S phase [Johnson et al. 1993]. Taken together, these studies suggest that E2F1 is a critical regulator of DNA synthesis.

Understanding the mechanisms by which E2F1 levels are regulated will provide insight into the complex signal

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transduction pathway that controls cell cycle progression. Using serum stimulation of quiescent cells as our model system, we have shown that the increase in E2F1 mRNA at the G1/S-phase boundary is abolished by a protein synthesis inhibitor (Slansky et al. 1993), suggesting that E2F1 expression is controlled by a transcription factor that is absent in quiescent cells. To test whether E2F1 is regulated by changes in promoter activity, we have cloned and sequenced E2F1 genomic DNA. Here, we show that the mouse E2F1 promoter can be activated by E2F proteins, that transcription from this promoter is growth-regulated, and that at least two elements are required for maximal transcriptional stimulation at the G1/S-phase boundary.

Results

Cloning of mouse E2F1 genomic DNA

Two different phage clones containing E2F1 DNA were isolated from a mouse genomic library [see Materials and methods for a complete description of the cloning]. One clone, mE2F1-892, was identified using the DNA-binding domain of the E2F1 cDNA as a probe and contained an insert of ~21 kb. Sequence analysis using primers derived from various regions of the E2F1 cDNA indicated that this clone did not contain the 5' end of the cDNA. However, a 2.4-kb Xhol-BamHI fragment from mE2F1-892 did contain three exons (Fig. 1A). Two of these exons, corresponding to E2F1 amino acids 82 to 110 and 111 to 184, contained the DNA-binding domain. A second clone, mE2F1-0315, was subsequently identified using sequences located near the 5' end of the E2F1 cDNA and contained a 19-kb insert; Southern blot analysis confirmed that the 5' end of the E2F1 cDNA was in a 4.7-kb SacI fragment of this clone.

Identification of the mouse E2F1 promoter

As a first step toward identifying the E2F1 promoter, we used primer extension analysis with two different primers to determine the distance from the 5' end of the cloned cDNA to the transcription start site [Fig. 2A]. On the basis of our previous work, we expected E2F1 mRNA to be low in serum-starved cells and increase at the G1/S-phase boundary (Slansky et al. 1993). The size of the extension products detected by either primer in RNA prepared 12 hr after serum stimulation was consistent with a transcription start site 29 bp upstream of the 5' end of the cloned cDNA [Fig. 2A]. Although this indicated that the cDNA was almost full length, we could not rule out the presence of an intron within the first 29 nucleotides of the transcript. Attempts to perform S1 or RNase protection assays were not successful, perhaps because of the high G+C content of the 5' end of the E2F1 gene.

Figure 1. Mapping exons 1–4 of the mouse E2F1 gene. (A) Southern blot analysis of the mE2F1-892 genomic clone indicated that a 2.4-kb Xhol-BamHI fragment contained exon sequences [■]. Three exons were defined by sequence analysis, two of these exons correspond to the basic region and helix-loop-helix domain of the E2F1 protein. NT (nucleotide), aa (amino acid). (B) Southern blot analysis of the mE2F1-0315 genomic clone indicated that a 4.7-kb SacI fragment contained exon sequences [■]. The 5' and 3' end of exon 1 were defined by sequence analysis and primer extension [Fig. 2]; the sequence of exon 1 is shown in Fig. 3.
Mapping the E2F1 transcription initiation site. (A) Primer extension of cellular RNA. Primer extension analysis was performed on RNA prepared from serum-starved and -stimulated NIH-3T3 cells. S phase starts ~10 hr after serum stimulation (Means et al. 1992). The arrows indicate the extension products from the primers shown in the schematic at the bottom of the figure. The X’s indicate that both primers produce extension products 29 nucleotides longer than the 5’ end of the E2F1 cDNA. Primer sequences are shown in Fig. 3A. (B) Primer extension of in vitro-transcribed RNA. In vitro transcription reactions were performed with a fragment containing -176 to +98 of E2F1 plus additional plasmid sequences using HeLa or K562 cell nuclear extracts. The extension products and the primer are shown schematically at the bottom of the figure. Primer extension of the in vitro-transcribed RNA yielded a product that is 29 nucleotides longer than the 5’ end of the E2F1 cDNA. To confirm that the extension products were based on RNA polymerase II-initiated RNA, α-amanitin was added to the in vitro transcription reaction mixture.

Because splicing does not occur in this in vitro system, these results suggest that transcription of the E2F1 gene begins ~98 nucleotides upstream of the Xmal site that is 29 bp upstream of the end of the cloned mouse cDNA [only 1 nucleotide upstream from the 5’ end of a human E2F1 cDNA (Shan et al. 1992)].

Sequence analysis of the 274-bp Xmal fragment [Fig. 3A] indicates that the E2F1 promoter is very G+C rich (74% between -176 and +98), does not contain a consensus TATA box 30 bp upstream of the initiation site, but does contain potential binding sites for a variety of known transcription factors [Table 1]. Although simple sequence inspection cannot determine the functional significance of a binding site, CCAAT boxes and Sp1 sites are common elements of many promoters and thus we have indicated the position of these in Figure 3A.

Also indicated are the two consensus E2F sites located just upstream of the transcription start site, each of which overlaps a 7 out of 8 match to a consensus E2F site on the opposite strand. The mouse and human E2F1 promoters are remarkably similar in sequence [Fig. 3B]. In fact, the 173-bp region extending 5’ of the E2F1 transla-
Figure 3. The E2F1 promoter sequence. (A) The sequence of the mouse E2F1 5'-flanking region from -176 into intron 1 is shown (GenBank accession number U08995). The consensus Sp1 sites (square boxes), CCAAT sites (oval boxes), E2F sites (bold lines), 1-base mismatch E2F sites (dashed lines), translation start site (dotted lines), and XmaI sites shown in Fig. 1B are indicated. The orientation and locations of the primers [P01, P02, P03] used to screen the genomic library and to map the transcription initiation site are indicated by arrows. The transcription initiation site is indicated by a bent arrow. The transcription start site is indicated by a bent arrow. The 5' end of the mouse and human promoters suggested a functional role of E2F1 and E2F3 stimulate E2F1 transcription

The conservation of the multiple E2F sites in the mouse and human promoters suggested a functional role of these sites. To address this possibility, we first examined protein binding to these sites using a mobility retardation assay. Probes containing either the E2F sites from -20 to +9 of the dhfr promoter or the -45 to +7 region of the E2F1 promoter were incubated with Friend cell nuclear extract [Fig. 4A]. Both probes bound protein, and the binding was reduced with an excess of the wild-type (D), but not a mutated (M), dhfr oligonucleotide. Also, the E2F1 promoter fragment [E] could reduce protein binding to the dhfr promoter fragment. These results suggest that cellular E2F binds to the E2F1 promoter with approximately the same affinity as to the dhfr promoter. To determine if both of the E2F sites were capable of binding protein, oligonucleotides containing just the 5' E2F site (-44/-25) or just the 3' E2F site (-26/-7) were used as probes [Fig. 4B]. Competitions were performed using a wild-type E2F1 promoter fragment [W], a promoter fragment having a mutation in the 3' site (ΔS'), a promoter fragment having a mutation in the 5' site (ΔS'), or a fragment having both sites mutated (ΔΔS). Both individual sites bound protein as shown by the direct mobility retardation of either probe and by the ability of fragment containing either site alone to serve as a competitor. Mutation of both consensus sites (ΔΔS) abolished competition.

E2F is a multigene family (Helin et al. 1992; Kaelin et al. 1992; Girling et al. 1993; Ivey-Hoyle et al. 1993; Lees et al. 1993; Li et al. 1994) and the mobility retardation analysis did not distinguish which protein bound to the E2F1 promoter. To test whether different E2F family members could activate transcription from the E2F1 promoter, we cotransfected NIH-3T3 cells with plasmids that direct expression of either E2F1, E2F3, or DP-1 plus the E2F1 promoter fragment (E) could reduce protein bind-

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The expression of E2F1 activated the E2F1 promoter reporter about 100-fold relative to the appropriate vector control. The expression of E2F1 increased the activity of the E2F1 promoter about 30-fold, indicating that multiple E2F family members could be involved in the regulation of E2F1. Transfection of DP-1 did not significantly activate the E2F1 promoter (data not shown), probably because levels of DP-1 are not limiting in quiescent cells (Li et al. 1994). The inability of transfected DP-1 to activate transcription through E2F sites has been documented previously (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993). Thus, the E2F1 promoter contains sites that can bind cellular E2F protein and the E2F1 promoter can be activated by multiple E2F family members.
Table 1. Putative regulatory elements found in the 5'-flanking region of the mouse E2F1 gene

| Element | Consensus | Number | Location       |
|---------|-----------|--------|----------------|
| CAAT box | (CCAAT)   | 3      | [-166], [-116], [-73] |
| GC box | (GGCCG)   | 3      | [-155], [-77], +23 |
| AP-2 1. | (YCCSMNS S S) | 1      | -109 |
|         | (TCCGGAT CGG) |        |               |
| AP-2 2. | (GSGW G S CC) | 2      | [-161], [+58] |
|         | (GGGTGG CC) |        |               |
| CRE     | (KWCGTCA) | 1      | -92 |
| E2F     | (TTTSCGC) | 2      | [-29], -23 |
| YY1     | (TGCGGA) | 1      | [-104] |
| Adf-1   | (CGCCGCNGC CGNCT) | 1      | +74 |
| PuF     | (GGGTCGCG) | 1      | [-143] |

The sequence from -176 to +98 was searched with the NIH database of transcription factor-binding sites (v. 7.0). Only those sequences showing a perfect match to a consensus RNA polymerase II transcription factor-binding site are reported; sites have been grouped based on the most commonly used name of the factor. A comparison of each site to the consensus site (in parentheses) is shown. Brackets indicate that the site is present on the opposite strand to that shown in Fig. 3A.

Although E2F sites are required for activation by E2F1, not all promoters that contain consensus E2F sites can be activated by this transcription factor in our transfection system [Li et al. 1994]. To test whether sequences besides the E2F consensus might be required for optimal activation, we analyzed several different E2F1 promoter constructs in the cotransfection assay [Fig. 4D]. In particular, because the 5'-untranslated region is highly conserved between mouse and human E2F1, we tested the effects of deleting a portion of this region. We found that deletion of the +36 to +98 region greatly reduced the activation by cotransfected E2F proteins. Fragments spanning either -45 to +36 or -176 to +36, each containing the consensus E2F sites, were only sufficient for low-level activation [12- to 16-fold] by E2F1. Mutation of the E2F sites in the -176 to +36 construct abolished activation by E2F1 (data not shown), demonstrating that the E2F1 directly activated transcription through these sites. However, because no E2F sites are present from +36 to +98, our results also suggest an indirect activation by E2F1 through this region. Alternatively, E2F proteins could bind to nonconsensus E2F sites to activate via the +36 to +98 region.

Growth-regulated transcription of the mouse E2F1 promoter

Primer extension analyses indicated that transcripts initiating at the +1 start site increase at the G1/S-phase boundary [Fig. 2A]. Using a serum starvation and stimulation assay, we asked if the -176 to +98 promoter fragment contained the cis-acting elements responsible for the growth-regulated expression of E2F1 [Fig. 5A]. Activity from the -176/+98 E2F1 promoter construct increased 84-fold by 12 hr after serum stimulation [Fig. 5A], suggesting that the observed changes of E2F1 message level during the growth cycle are the result of the transcriptional regulation of the E2F1 promoter. Because analysis of a -176/+36 promoter construct showed a 42-fold activation [Fig. 5B], we used this construct for further mutational analysis of the E2F1 promoter. To determine if the E2F sites were necessary for growth-regulated transcription, we mutated either the proximal E2F site (A3'E2F) or both E2F sites (AAE2F) in the -176 to +36 construct. Growth regulation from the construct in which the E2F sites were mutated was reduced from 42- to only 5-fold, indicating that the E2F sites are critical for maximal regulation. To determine whether the sequences containing the E2F sites were sufficient for growth-regulated transcription, we tested a fragment spanning from -45 to +36, which contains the E2F sites and the transcription initiation site. This fragment was not sufficient for growth-regulated expression, suggesting that an element critical for growth regulation was present between -176 and -45. However, when the -176 to -37 region was inserted upstream of a synthetic promoter [-176/+36TI], it could not confer a large increase in activity at the G1/S-phase boundary.
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**Figure 4.** E2F family members bind to and activate transcription from the E2F1 promoter. **(A)** Comparison of protein binding to the E2F site in the −20 to +9 dhfr promoter region to the −45 to +7 E2F1 promoter region. Mobility retardation analysis was performed using Friend cell nuclear extract and wild-type dhfr oligonucleotide (D) of the sequence CTAGCAGCTGCTGCGATTTCGCGC-CAAACTTGACG, mutated dhfr oligonucleotide (M) of the sequence CTAGCAGCTGCTGCGATTTCtCGCaAAACTTGACG, or wild-type E2F1 oligonucleotide (E) competitors. The positions of the probe and shifted bands are indicated by arrows. **(B)** Comparison of protein binding to the 5′ and 3′ E2F sites of the E2F1 promoter. Mobility retardation analysis was performed using Friend cell nuclear extract and an oligonucleotide containing either the 5′ or the 3′ E2F site. Fragments containing both sites (W), a mutation in the 5′ site (Δ5′), a mutation in the 3′ site (Δ3′), or mutations in both sites (ΔΔ) were used as competitors. The amount of competitor and the positions of the probe and shifted bands are indicated. **(C)** The E2F1 promoter can be activated by E2F1 and E2F3 in quiescent cells. NIH-3T3 cells were transfected with 5 μg of E2F1 (−176/+98)–Luc and variable amounts of pCMV-mE2F1, pCMV-E2F-3, or pcDNA3 (vector control). After transfection, the cells were starved in medium containing 0.5% calf bovine serum for 48 hr before harvesting for luciferase assays. The fold induction of the E2F1 promoter was calculated as the ratio of the luciferase activity in the presence of the E2F expression plasmid to the activity in the presence of the vector control. The data shown are an average of duplicates from one experiment and can be compared to an average of multiple experiments shown in Fig. 4D. **(D)** Maximal activation by E2F requires multiple DNA elements. Each E2F1 promoter reporter construct was transfected with different amounts of E2F1 or E2F3 expression constructs as in Fig. 4C. The maximal activation obtained is reported; each bar is an average of three experiments with at least two DNA preparations.

These results suggested that at least two regions are required for growth-regulated expression of the E2F1 promoter: the E2F sites and elements within the −176 and −45 region.

We then made 5′ deletions to delineate the upstream sequences important for growth-regulated transcription. Constructs having 5′ ends at −125 or −84 were still growth responsive, showing 22- or 14-fold increases in activity 12 hr after serum stimulation. Because further deletion to −45 had resulted in a complete loss of regulation, sequences between −45 and −84 must be critical for growth-regulated expression from the E2F1 promoter.
The -45 to -84 region is 70% identical between mouse and human E2F1, contained within this region of the mouse E2F1 promoter are consensus binding sites for Sp1 and CCAAT factors, but only the CCAAT site is conserved in the human promoter. To determine whether these sequence elements bound protein, oligonucleotides containing either Sp1 or CCAAT boxes were used in mobility retardation assays (Fig. 5C). The CCAAT probe bound protein, and the binding was abolished with a 20-fold excess of a fragment containing the wild-type [W] sequence element. The Sp1 site also bound protein, and competition was abolished after addition of the wild-type [W] fragment as a competitor. However, a promoter fragment having a mutation of the Sp1 site located at -77 still competed binding. We note that the E2F1 promoter fragment contains a second consensus Sp1 site located at +23, unlike the -77 Sp1 site, the site at +23 is conserved between the mouse and human E2F1 promoters (Fig. 3B). Therefore, mutation of the -77 Sp1 site did not prevent Sp1 binding to the E2F1 promoter. Promoter constructs containing a mutated -77 Sp1 site or a mutated CCAAT box were then tested.
in the serum starvation and stimulation assay (Fig. 5B). Mutation of the Sp1 binding site did not reduce transcriptional activity (data not shown) or regulation (Fig. 5B), perhaps because of the other functional Sp1 site at +23. Mutation of the CCAAT site reduced, but did not abolish, regulation. These results suggest that different elements can confer relatively high (CCAAT = 14-fold) or moderate (Sp1 = 6.5-fold) growth regulation on the nonresponsive −45 to +36 promoter region. However, as shown by the lack of regulation of the −176/−37TI construct, these elements must cooperate with the −45/+36 region to confer growth-regulated transcription.

Although both the E2F and the CCAAT mutant constructs reduce the fold increase in activation at the G1/S-phase boundary, the effects of these mutations on promoter activity is quite distinct, as seen by examination of the promoter activity from individual constructs (Fig. 6). Mutation of the E2F sites increased promoter activity in quiescent cells by −fivefold but had little effect on the S-phase value (Fig. 6A). In contrast, mutation of the CCAAT site had little effect on basal activity but reduced the S-phase value by −fourfold (Fig. 6B). Thus, the E2F1 promoter is regulated by both E2F-site-mediated repression in G0 phase and CCAAT- or Sp1-site-mediated activation in S phase.

Discussion

We have cloned the mouse E2F1 promoter and demonstrated functional activity using in vitro nuclear extracts and transfection into NIH-3T3 cells. Our previous work has shown that mouse E2F1 mRNA levels increase at the G1/S-phase boundary [Slansky et al. 1993]; we now demonstrate similar changes in transcriptional activity of the E2F1 promoter. We have developed a model (Fig. 7) for the regulation of the E2F1 promoter that accounts for three observations:

1. The E2F1 promoter contains E2F-binding sites that are necessary, but not sufficient, for growth-regulated transcription

Mutation of the E2F sites created a promoter whose activity increases only fivefold at the G1/S-phase boundary, indicating that the E2F sites are important for maximal growth-regulated transcription of the E2F1 promoter. The decreased regulation was the result of an increase in activity of the E2F1 promoter by three- to fivefold in quiescent cells, suggesting that activity from this promoter is repressed in G0 via E2F sites. Mutation of E2F sites has resulted in a varying degree of promoter activation in previous studies. For example, mutation of the E2F sites in the mouse dhfr promoter results in less than a twofold increase in activity in quiescent cells [Means et al. 1992]. In contrast, mutation of the E2F sites in the B-myb promoter results in a 10-fold increase in activity in quiescent cells [Dalton 1992]. These results suggest that different promoters may be regulated differently by E2F family members. Alternatively, the E2F1 and dhfr promoters may contain binding sites for additional G0-specific repressors or specific cell type or culture condi-
tions may enhance the degree of repression seen in other studies.

Our finding that the E2F sites in the -45 to +36 region of the E2F1 promoter were not sufficient for growth-regulated expression was surprising in light of our previous studies with the dhfr promoter. Two copies of an oligonucleotide containing the dhfr E2F sites show a 35-fold increase in promoter activity at the G1/S-phase boundary [Slansky et al. 1993]. The different sequences and orientations of the sites in the dhfr and E2F1 promoters may account for the different results. Alternatively, the dhfr -20 to +9 sequences may contain an unidentified activator that is critical for the observed regulation.

2. Sequences in the E2F1 promoter upstream of the E2F sites are necessary, but not sufficient, for growth-regulated transcription

Deletion of sequences between -84 and -45 abolish high-level expression in S phase, suggesting that the E2F1 promoter is regulated by activation in S phase as well as by repression in G0. Sequences from -453 to -348 of the B-myb are also necessary for high expression in S phase [Lam and Watson 1993]. Similarly, upstream sequences of the dhfr promoter contribute to S-phase activation [Farnham et al. 1993]. Insertion of the -176 to -37 region of the E2F1 promoter upstream of a synthetic promoter does not result in high promoter activity, suggesting that high transcriptional activity requires an interaction between elements in both the -84/-45 and the -45/+36 regions. Contained within the -84 to -45 region are Sp1 and CCAAT boxes; only the CCAAT box is conserved between the human and mouse E2F1 promoters. CCAAT-box-binding proteins have been implicated in the growth regulation of other promoters such as thymidine kinase [Pang and Chen 1993] and hsp70 [Lum et al. 1992]. Contained within the second region required for growth regulation (-45 to +36) are E2F sites and an Sp1 site at +23. Sp1 sites in the fos, insulin-like growth factor II, and neu promoters [Kim et al. 1992; Yu et al. 1992; Udvadia et al. 1993] have been implicated in transcriptional regulation by the retinoblastoma (Rb) tumor suppressor protein. Sp1 has been shown to interact with itself, as well as with a variety of other transcription factors [Janson and Pettersson 1990; Li et al. 1991; Mastrangelo et al. 1991; Hoey et al. 1993; Lee et al. 1993]. Mutation of the CCAAT and Sp1 sites in the context of the -176/+36 E2F1 promoter construct may aid in understanding the protein–protein interactions required for S-phase activation.

3. The E2F1 promoter can be activated by E2F1 protein by >100-fold

This enormous activation contrasts that of most E2F site-containing promoters that are usually activated on the order of 5–10-fold by E2F1 in our transfection system [Li et al. 1994]. Deletion of sequences between +36 and +98 [but leaving all the consensus E2F sites] reduces the stimulation of the E2F1 promoter by the E2F1 expression construct to 12-fold. This remaining activation is abolished by mutation of the E2F sites in the -176/+36 construct (data not shown). Because there are no E2F sites between +36 and +98, E2F1 may mediate activation through this region by an indirect mechanism, for example, by increasing the levels of another transcription factor. The deleted region contains consensus binding sites for two transcription factors, Adf-1 [at present only identified in Drosophila cells] and Ap2. Mutational analysis is required to determine which, if either, of these sites is responsible for the high-level activation of the E2F1 promoter. Deletion of the +36 to +98 region only reduces the G1/S-phase activation by about twofold. The differences in requirement for this region in trans-activation by E2F1 family members versus G1/S-phase regulation could be the result of differences in the assay conditions: the cotransfections are performed in serum-starved cells, whereas the growth regulation is analyzed after the addition of serum.

In summary, we find that multiple DNA elements regulate the E2F1 promoter via repression in G0 and activation in S phase [Fig. 7]. Our working model is that an E2F site located near the -30 region prevents productive transcription complex formation on the E2F1 promoter in quiescent cells. E2F may interfere with formation of this complex by simply occluding binding of TFIIID to the -30 region or by bringing in an “active” repression domain via interactions of E2F family members with Rb or p107 [Dalton 1992; Hamel et al. 1992; Weintraub et al. 1992]. The second possibility is favored because studies showing E2F–TBP interactions [Hagemeier et al. 1993] suggest that free E2F could help tether TFIIID to the DNA. The increase in levels of free E2F proteins, either by transfection of family members into quiescent cells or by changes in protein–protein interactions in late G1 [Bandara et al. 1991; Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Lees et al. 1992; Nevins 1992; Pagano et al. 1992; Shirodkar et al. 1992; Chittenden et al. 1993], may relieve the repression from the E2F1 promoter, producing more free E2F1 and setting up a positive autoregulatory loop that results in an extremely high activation. However, efficient formation of a productive transcription complex may require binding sites for strong activators both upstream (e.g., Sp1 or CCAAT) and downstream (e.g., Sp1) of +1. Thus, both activation and repression play critical roles in E2F1 regulation: deletion of the activator binding sites reduces regulation from 42- to 1-fold, and relief of repression by mutation of the E2F sites reduces regulation from 42- to 5-fold. It remains unclear exactly which E2F family members are involved in the repression of the E2F1 promoter. Future studies of the individual E2F proteins and the role of different protein–protein interactions will address various aspects of this model.

Materials and methods

Library screening

A mouse EMBL-3 genomic library [Clontech] in Escherichia coli Y1090 was plated at 3 × 10⁶ plaques per 150-mm-diam. dish and
duplicate phage lift filters were made from each plate. The filters were incubated in 0.5 M NaOH for 90 sec, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 min, rinsed in 3× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), then baked at 80°C for 2 hr. When the probe used was a DNA restriction fragment, hybridization was as described (Li et al. 1994). After hybridization, the filters were washed twice at room temperature (for 15 min and then for 45 min) in 2× SSC, 0.1% SDS and then at 37°C for 30 min in 0.2× SSC, 0.1% SDS. When the probe used was a small synthetic oligonucleotide, the filters were prehybridized for 3 hr at 30°C in 6× SSC, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone), 50% formamide. 0.5% SDS, 100 μg of sonicated salmon sperm DNA per ml. Hybridization was performed at 30°C in the same solution for 16 hr. The oligonucleotides were 5' end-labeled with [γ-32P]ATP using T4 polynucleotide kinase, purified through a Sephadex G-50 column, and added directly to the hybridization mix. After hybridization, the filters were washed at room temperature three times for 15 min in 2× SSC, 0.1% SDS, and then at 50°C for 2 hr in 0.2× SSC, 0.5% SDS. In all cases, the hybridization signals were visualized by autoradiography. Plaques that were positive on duplicate filters were isolated and purified by additional rounds of screening.

Using an E2F1 cDNA fragment encoding amino acids 82 to 205 as a probe, we obtained one positive clone after screening 1× 10^9 plaques. This clone, mE2F1-892, contained internal exons of mouse E2F1 [Fig. 1A] but could not be sequenced by primers complementary to the 5' end of the E2F1 cDNA. We then used an oligonucleotide (P03, see Fig. 3A) derived from the 5' end of the mouse cDNA as probe, and one positive clone was obtained after screening 1×10^6 plaques. Characterization of this clone, designated mE2F1-0315 [Fig. 1B], indicated that it contained exon 1 of the mouse E2F1 gene. Thermal cycle sequencing using E2F-specific primers indicated that the 19-kb insert does not extend to exon 2 (data not shown).

Subcloning and sequencing

Phage DNA was purified from plate lysates using the Magic Lambda DNA purification system [Promega]. A 2.4-kb XhoI-BamHI fragment [which hybridized to the E2F1 cDNA in Southern blot analysis] from the 21-kb insert of mE2F1-892 was subcloned into pBluescript II KS (Stratagene), generating pXB2.4. Deletion constructs from the Xhol site of pXB2.4 were prepared using an Erase-A-Base system [Promega] and sequenced by the dideoxynucleotide chain termination method using Sequenase 2.0 [US Biochemical] and vector primers. By comparison to the E2F1 cDNA, this subclone contained exons 2, 3 and 4, primers based on the cDNA could not identify exon 1 in pXB2.4 or in clone mE2F1-892. A 4.7-kb SacI fragment [which could hybridize with P01 in Southern blot analysis] from the 19-kb insert of mE2F1-0315 was subcloned into pBluescript II KS generating pE2FI-1.7. A 274-bp XmaI–XmaI fragment derived from pE2FI-1.7 was inserted into the XmaI site of pBluescript II KS generating pE2FI-0.27 and into the XmaI site of pGL2Basic [Promega] generating pE2FI[−176/+36]Luc. The complete sequence of both strands of the 274-bp XmaI fragment was determined, an additional 283 bp 3' of the XmaI site in pE2FI-1.7 was also sequenced using primers derived from cDNA sequence to define the 3' end of exon 1.

Smaller E2F1 promoter constructs were generated from pE2FI-0.27 by polymerase chain reaction using Taq polymerase [Promega] with primers specific for E2F1 sequences and vector primers. The amplified products were cloned into either the XmaI–BglII sites of pGL2Basic generating pE2FI[−176/+36]–Luc and pE2FI[−45/+36]–Luc or the XmaI site generating pE2FI[−45/+98]–Luc. A promoter construct containing a mutation in the 3' E2F site was prepared as follows. PCR was performed on pE2FI-0.27 using a T3 primer and a primer specific to E2F1 sequences from −6 to −28 (ntemplate strand) except containing an EcoRI site instead of the proximal EcoRI site to produce sequences from −176 to −6 Δ3'E2F. A second PCR reaction was performed on pE2FI[−176/+36]–Luc using a luciferase primer and a primer specific to E2F1 sequences from −6 to −28 (template strand) except containing an EcoRI site instead of the 3' E2F site to produce sequences from −28 to +36 (Δ3'E2F). The products were purified, digested with EcoRI, ligated, cut with Xmal and HindIII, and cloned into Xmal–HindIII cut pGL2 Basic to generate −176/+36Δ3'E2F. The sequence from −28 to −6 was now AAGGATTTGaattcTAA-AAGTGCG [mutated bases are in small letters]. A construct with the 5' E2F site mutated [used only in the mobility retardation analysis] was created similarly except that the primers contained sequences from −45 to −23 with a PstI site instead of the 5' E2F site. The sequence from −45 to −23 was now GCC-TCTTTTcgcagAAAAAGGAT [mutated bases are in small letters]. A construct with both E2F sites mutated (−176/ +36Δ2E2F) was created using a single-site mutation construct as a template. The E2F1 promoter constructs pE2FI[−125/+36]–Luc and pE2FI[−84/+36]–Luc were generated by PCR from pE2FI[−176/+36]–Luc using E2F1-specific primers and a primer specific for the luciferase cDNA. The amplified products were digested with Xhol and HindIII and cloned into Xhol–HindIII cut pGL2Basic. pE2FI[−176/+37]–TILuc was made similarly except that the amplified product was inserted into the Xmal–XhoI site of pTILuc [Miltenberger et al. 1993]. pE2FI[−125/+36CAT]–Luc, pE2FI[−84/+36SP1]–Luc, and pE2FI[−84/+36CAT]–Luc were constructed in a similar manner except that the E2F1-specific primers contained mutations in the CCAAT or SP1 consensus sites near the deletion endpoint (the exact mutations are described for the mobility retardation assays).

Mapping the E2F1 transcription initiation site

Cytoplasmic RNA (20 μg) from NIH-3T3 cells synchronized by serum starvation and stimulation was prepared as described [Slansky et al. 1993] and hybridized to primers [shown in Fig. 3A] complementary to E2F1 mRNA. Primer extension reactions were performed as described [Farnham and Kollmar 1990]. In vitro-transcribed RNA was produced using nuclear extract from human K562 or HeLa cells. HeLa cells were grown, and extract was prepared as described [Means and Farnham 1990]. K562 cells were grown in RPMI 1640 [Life Technology], supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin [Life Technology], and 5% [vol/vol] defined/supplemented bovine calf serum [Hyclone Laboratories], and nuclear extract was prepared as described [Means and Farnham 1990], except that the cells were lysed in 1 mM KOAc, 1.5 mM MgAc, 10 mM Tris (pH 7.6), 2 mM DTT, 100 μg/ml of PMSF, 100 μM EGTA instead of the standard buffer A [Dignam et al. 1983]. In vitro transcription reactions and primer extensions were performed as described [Means and Farnham 1990], except that 10 nm of a PvuII fragment from pE2FI-0.27 was used as template and the P01 primer was hybridized at 55°C. In some in vitro transcription reactions, α-amanitin [2.5 μg/ml of final concentration] was added to the initial incubation.

Cell culture and transient transfections

NIH-3T3 cells [American Type Culture Collection] were maintained, transfected, and stimulated with serum as described.
Mobility retardation experiments

E2F DNA-binding activity was assayed by incubating 6 μg of Friend cell nuclear extract with 2 μg of sonicated salmon sperm DNA in a total volume of 14 μl containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl₂, 0.4 mM DTT, 0.08 mM EGTA, 4% Ficoll, 0.04% NP-40 for 10 min at room temperature. Probe (0.4 ng) in 6 μl of water was then added, and the incubation continued for 15 min. The reactions were electrophoresed on 10% polyacrylamide gels that had been preelectrophoresed for 60 min. The gel buffer was 0.25× TBE (Maniatis et al. 1982). The probe was either a 30-bp double-stranded oligonucleotide spanning from −20 to +9 of the mouse dhfr promoter or a 69-bp double-stranded oligonucleotide spanning from −45 to +7 of the mouse E2F1 promoter, or 20-bp oligonucleotides spanning from −44 to −25 or −26 to −7 of the mouse E2F1 promoter. The probes were phosphorylated with T4 polynucleotide kinase and [γ-32P]ATP (Maniatis et al. 1982). When competition assays were performed, the competitor DNA was included in the first incubation. Competitor DNAs used in Fig. 4A were either the E2F1 or dhfr oligonucleotides or a mutant dhfr oligonucleotide [mutant E/F] in Means et al. [1992] that has been shown previously to be nonfunctional. Competitor DNAs used in Fig. 4B were fragments spanning from −176 to +36 of the wild-type E2F1 promoter (W), from the promoter containing a mutation in the 5′ E2F site (Δ5′), from the promoter containing a mutation in the 3′ E2F site (Δ3′), or from the promoter having both sites mutated (ΔΔ). CCAAT-binding activity was assayed [as described by Pang and Chen (1993)] by mobility retardation using a double-stranded oligonucleotide containing sequences from −76 to −66 of the E2F1 promoter, flanked by additional sequences containing restriction enzyme sites. For competitions, Xhol–HindIII fragments were isolated from the −84/+36 or −84/+36ΔCAT promoter constructs. The CCAAT box was changed to AATTCC in the −84/+36ΔCAT construct. Sp1 binding activity was assayed using the conditions of Pang and Chen (1993) and a double-stranded oligonucleotide spanning from −84 to −64 of the −84/+36ΔCAT construct, plus flanking sequences containing a restriction enzyme site. This 20-bp oligonucleotide contains the Sp1, but not the CCAAT, site. For competitions, Xhol–HindIII fragments were isolated from −84/+36 or −84/+36ΔSp1 promoter constructs. The Sp1 site was changed from CCGCCG to GAATTCC in the −84/+36ΔSp1 construct. Friend nuclear extracts were prepared as described above for K562 extracts except that the Friend cells were grown in RPMI 1640, supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1×10⁻³ M β-mercaptoethanol, and 10% (vol/vol) defined supplemented bovine calf serum (Hyclone Laboratories).

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