Constitutive Protection of E2F Recognition Sequences in the Human Thymidine Kinase Promoter during Cell Cycle Progression*

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The sequences responsible for S phase-specific induction of the human thymidine kinase (TK) gene have been mapped to a small region that contains putative E2F binding sites. We have analyzed protein-DNA interactions at the TK promoter during cell cycle progression in human fibroblasts using an in vivo footprinting approach. We found 14 protein binding sites that were occupied in vivo. All of the sites (among them two inverted CCAAT boxes and several Sp1 sites) bound transcription factors constitutively throughout the cell cycle, i.e. none of the factor binding was cell cycle-dependent. An E2F-like site located between nucleotides −97 and −89 relative to the major transcription start site was protected in G0, G1, S, and G2 phases. This cell cycle-independent protection of E2F sequences in the TK promoter differs from the G0/G1-restricted binding of E2F complexes observed for genes in which the E2F sites function as repressor elements (Tommassi, S., and Pfeifer, G. P. (1995) Mol. Cell. Biol. 15, 6901–6913; Zwicker, J., Liu, N., Engeland, K., Lucibello, F. C., and Müller, R. (1996) Science 271, 1595–1597). A comparison of several genes containing E2F motifs indicates that E2F sites located in proximity to the transcription initiation site (−50 to +20) in TATA-less promoters predominantly function as repressor elements, while in other genes constitutively bound E2F complexes located further upstream mediate activation presumably in conjunction with a functional TATA box.

A large number of reports document the pivotal role that the transcription factor E2F may play in coordinating transcription during the mammalian cell cycle, particularly in the induction of specific genes at the G1/S transition (1–4). E2F was initially identified as a sequence-specific DNA-binding protein complex required for the E1A-dependent transcription of the adenovirus early E2 gene (5). Five distinct members of the E2F family (E2F-1 through E2F-5) have so far been identified (3, 4). These transcription factors heterodimerize in vitro with different DP proteins (DP-1 through DP-3) and this interaction appears to be essential for both high affinity DNA binding and E2F-dependent transactivation (6–8).

A number of proliferation-associated genes such as those encoding c-myc, B-myb, DNA polymerase α, dihydrofolate reductase, thymidine kinase (TK), cyclin A, cdc2, and E2F-1 contain potential recognition sites for E2F in their promoters and these sequences are important for cell cycle regulation of these genes (9–17). The presence of E2F binding sites within the promoters of several genes coding for DNA synthesis enzymes suggests that growth regulation of these proteins may take place by a common mechanism.

E2F sites can function as transcriptional activator or repressor elements which may be dependent on the stage of the cell cycle (18). This dual role as an activator and/or repressor is perhaps also a function of the promoter in which the E2F sites reside or the nucleotide sequence of the E2F site. In vivo footprinting studies have shown that E2F complexes can be released from their promoter binding sites upon induction of certain cell cycle-regulated genes during cell cycle progression (17, 19). Evidence from in vitro studies suggests that E2F complexes lose their DNA binding activity through kinase-mediated phosphorylation of the E2F protein by a cyclin A-Cdk2 complex (20–22). It is therefore of interest to investigate whether the release of E2F complexes is a feature of all E2F-regulated genes or whether this release may depend on promoter context or the functional role of the E2F complexes.

The human thymidine kinase gene is cell cycle regulated at the transcriptional level. Using deletion and site-directed mutational analysis, it was established that the upstream sequences of the human TK promoter, spanning nucleotides −133 to −64, designated CCRU (cell cycle regulatory unit), were sufficient to confer cell cycle regulation (23, 24). Furthermore, this 70-bp CCRU domain is able to confer G1-S regulation onto a non-cell cycle-regulated, heterologous promoter (24). This sequence includes the distal CCAAT box element, and three GC-rich motifs that resemble a consensus E2F binding site. Mutations of these elements almost completely abolished the promoter activity (25). In addition, one of the regulatory sites in the mouse TK promoter (MT2, −81 GTTCGCGGGCAAATG), is an E2F-like binding site that interacts with a fusion protein containing human E2F and mouse nuclear protein complexes containing E2F, p107, Cdk2, cyclin A, and cyclin E in a cell cycle-dependent manner (26).

For these reasons, a member of the E2F family can be considered a likely candidate for cell cycle regulation of the human thymidine kinase gene. However, despite the analogy with murine E2F-related cis-regulatory elements and the discovery that complexes containing cyclin A, p107 and p33cdk2 associate with a 25-bp long sequence inside the regulatory region, the involvement of E2F in human TK regulation is unclear (27–30).

In this study, we investigated the in vivo occupancy of the various cis-regulatory elements within the TK promoter region,
especially in relationship to the involvement of E2F in proliferation control. The 70-bp CCRU and 100 nucleotides of the surrounding sequences on both sides including the transcription initiation site were analyzed throughout the cell cycle with a high resolution in vivo footprinting method (31, 32). Our data provides direct evidence that the occupancy of this E2F site is constitutive throughout the cell cycle.

MATERIALS AND METHODS

Cell Culture and Synchronization—Conditions for culturing and synchronization of normal human foreskin fibroblasts (strain HF-39) have been described previously (17). Cells were brought to quiescence by serum deprivation for 14 days, then stimulated to proliferate by the addition of fresh medium (Dulbecco’s modified Eagle’s medium) containing 15% fetal calf serum and harvested at various time points.

Cell Cycle Analysis—HF-39 fibroblasts were serum-stimulated after 14 days of serum starvation and cell synchrony was monitored by flow cytometric analysis. Growth-arrested and serum-stimulated fibroblasts were stained with propidium iodide as recommended by the supplier (Becton Dickinson, San Jose, CA). Samples were run on a FACS IV (Becton Dickinson) flow cytometer. Data was acquired and analyzed on a Cicerone Workstation (Cytomation, Ft. Collins, CO).

RNA Preparation and Northern Blot Analysis—Subconfluent HF-39 fibroblasts were rendered quiescent by incubation in serum-free Dulbecco’s modified Eagle’s medium for 14 days. After addition of 15% fetal calf serum, cells were collected at the times indicated, and total RNA was isolated by a guanidine isothiocyanate method (RNAGentsTM, Promega). A 10-μg aliquot of each RNA sample was separated on a formaldehyde-agarose gel and transferred to a GeneScreen (NEN Life Science Products) nylon membrane. The membrane was then hybridized at 60 °C in 0.25 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA, 1% bovine serum albumin with a probe specific for the 7th exon of the human TK gene. A probe specific for the GAPDH gene was used as a control.

Genomic Footprinting with Dimethyl Sulfate (DMS)—Immediately after the serum starvation period and at different time points after serum substitution, subconfluent fibroblasts were treated with 0.2% DMS, and nuclei were isolated as described previously (17). DNA was isolated from pelleted nuclei and cleaved at methylated bases with hot piperidine. The chemically cleaved DNA was then amplified by ligation-mediated PCR (LM-PCR), the sequence ladders were separated on 8% acrylamide, 7 M urea gels in 0.1 M Tris-borate-EDTA, and transferred to nylon membranes (33, 34). 32P-Labeled single-stranded hybridization probes were synthesized by a PCR-based technique using primer 3 (see below) from the appropriate primer set (35, 36). G, G + A, C + T, and C reactions were obtained by in vitro treatment of purified DNA (either from fibroblasts or HeLa cells) with DMS (37). Four gene-specific LM-PCR primer sets, selected with the aid of a computer program (38), were used to analyze the promoter region of the human TK gene and are listed below. Primers a1, b1, c1, and d1 were used for primer extension with Sequenase, primers a2, b2, c2, and d2 were used for PCR amplification analysis, and primers a3, b3, c3, and d3 were used to make hybridization probes.

For analysis of the lower strand by LM-PCR, the following primers were used: tkα1, CCCGATCAGCCACGTC, Tm = 49.5 °C; tkα2, CCA- GGTCATGCCTGATTTCC, Tm = 62.2 °C; tkα3, ATTTCCAGGC- CTTCCAGTCC, Tm = 60.0 °C; tkβ1, GAACCTTGCTTGGGCAAA, Tm = 51.0 °C; tkβ2, CCACACAGACACATCCATGCGGCT, Tm = 62.5 °C; and tkβ3, ATCATGGCGTCTACAGCCGCATGG, Tm = 61.0 °C. For analysis of the upper strand, the following primers were used: tkc1, CGGGAGTTCCTCACGGAAC, Tm = 48.0 °C; tkc2, GTTTCAGAAC- CCCGAGTACTCTCCAG, Tm = 60.3 °C; tkc3, TCTTCAAGGCCTGCT- CCGGAGT, Tm = 64.5 °C; tkd1, GAGAGATTGGCGCCGAC, Tm = 53.8 °C; tkd2, CAGCCGGCGCCCTGGTGGG, Tm = 69.7 °C; and tkd3, ATTCGGGACGTGCGCCCAG, Tm = 65.7 °C.

UV Photofootprinting—Nonsynchronized fibroblasts and purified DNA from the same cells were irradiated with a germicidal UV lamp (30484 mW/cm2) at 37 °C for 30 min. DNA isolation, cleavage at cytosine/5-methyl cytosine dimers with T4 endonuclease V and photolysis, and ligation-mediated PCR analysis were done essentially as described previously (34).

RESULTS

Cell Cycle Synchrony—We have used normal human foreskin fibroblasts for serum starvation and cell synchronization experiments. We found that serum starvation for 48 h did not give a high degree of cell cycle synchrony. Serum starvation for longer periods of time (up to 14 days) drastically improved levels of synchrony after restimulation with serum. We deter-
majority of the cells are in S/G2. In asynchronously cycling HeLa cells, where transcriptional activation of the TK gene has been shown to be deregulated during the cell cycle (41, 42), much higher levels of TK mRNA are present compared with those in unsynchronized fibroblasts (Fig. 2, last two lanes).

**In Vivo Footprinting of the Human Thymidine Kinase Promoter**—To identify upstream regulatory elements, at which the binding pattern of transcription factors may change as a function of the cell cycle, we have investigated protein-DNA interactions in vivo at the TK promoter by a genomic footprinting technique. Human fibroblasts were treated with DMS, a methylating agent that reacts predominantly with guanines at the N-7 position, enabling later cleavage of the modified bases by hot piperidine. Sequence ladders were then amplified by LM-PCR and analyzed on sequencing gels as described (31–34, 43).

In a situation where transcription factors are bound to the DNA, they will either decrease accessibility of specific guanines to DMS (protection) or, often at the edges of a footprint, increase reactivity (hyperreactivity). When DMS patterns from naked DNA controls and patterns from serum-starved fibroblasts and cells at various time points following growth stimulation are compared, one might be able to detect protein-occupied sites and could therefore identify those elements at which changes in protein-DNA interactions occur during cell cycle progression. Four different LM-PCR primer sets were designed to analyze the 5'-flanking region of the human TK gene, beginning from approximately 300 bp upstream of the transcription initiation site.

First, we analyzed sequences between nucleotides −263 and −162 upstream of the transcription start site (Fig. 3). There are several potential Sp1 binding sites located within this region. Clear footprints were observed at two Sp1 consensus sites (5'-GGGC/CGG and 5'-GAGGC/CGG) located between nucleotides −232 and −227 and nucleotides −223 and −217, respectively. Additional footprints were seen near another potential Sp1 binding site (5'-GGGTC/CGG, nucleotides −202 to −196; confirmed by analysis of the opposite strand) and a sequence near nucleotide −185, which interacts with an unknown factor. These protein-DNA interactions were all constitutive throughout the cell cycle, i.e. there was no change in the binding pattern at the beginning of S phase (18 h).

Fig. 4A shows a genomic footprinting analysis of upper strand sequences spanning from nucleotides −7 to −137 relative to the transcription start site. This region contains a putative TATA box, at position −21, which deviates by 1 nucleotide from the canonical TATA site, and two inverted CCAAT boxes, at positions −36 to −40, and −67 to −71, respectively.

The two CCAAT elements have been reported to bind the transcription factor NF-Y (or CP1) in a cell cycle-specific manner (44–46), suggesting that the binding of CCAAT-binding proteins may regulate S phase-specific transcription of TK. Deletion of both CCAAT boxes severely impaired promoter strength and excision of the distal CCAAT box seemed to be more deleterious than excision of the proximal CCAAT box (47, 48). Both the proximal and distal CCAAT elements showed a similar in vivo footprint pattern (Figs. 4 and 5). The two guanines of the core motif are partially protected from DMS modification at all time points. To further confirm protein binding...
at the two CCAAT boxes, we have also used UV light for footprinting (see below; Fig. 5). The constant DMS modification pattern at the two guanines indicates that these two elements are occupied in vivo before serum addition and during all subsequent phases of the cell cycle (Fig. 4A). Thus, cell cycle-dependent binding of CCAAT box binding factors does not appear to be involved in TK gene regulation. Binding of these factors is constitutive, but may be regulated by posttranslational modification of promoter-bound factors.

Farther upstream, near the top of the gel, an area of protection is evident. This area corresponds to an Sp1 consensus binding site (5'-GGGCGG), and also this site is occupied before serum addition and during all subsequent phases of the cell cycle.

In Fig. 4B the same sequences were analyzed on the opposite DNA strand. The TATA box, the two CCAAT elements (which are not footprinted on the C-rich strand), one Sp1 site as well as one additional footprint are indicated. An area of strong DMS protection is seen at a potential E2F site. This sequence (5'-CCTGGCGGG, nucleotides -89 to -97; the Gs protected from DMS modification are underlined) resembles a typical E2F consensus binding site (5'-TTTSSCGC, where S is G or C), found in other G1-S-regulated promoters. Since this sequence is located inside the CCRU, members of the E2F family may be involved in cell cycle regulation of the TK gene. As shown in Fig. 4B, this footprint does not change as a function of the cell cycle, indicating that the E2F-like site is always occupied in vivo at various stages during cell cycle progression (G0, G1, S, and G2). Therefore signals other than sequence-specific binding to the DNA of the E2F-like factor must be involved in G1-S activation of the TK gene.

To further confirm protein DNA interactions at the two CCAAT boxes which were only partially protected from DMS modification in vivo (Fig. 4A), we have conducted a UV photoprotein analysis (Fig. 5). Fibroblasts were UV-irradiated and the distribution of cyclobutane pyrimidine dimers was mapped and compared with that in irradiated naked DNA (34). The UV photoproduct patterns are only slightly different between UV-irradiated naked DNA and UV-irradiated cells at the E2F binding site. However, there were two very conspicuous in vivo UV hyperreactive sites located at the TT sequences of both inverted CCAAT boxes. This hyperreactivity for pyrimidine dimer formation has been observed previously at other CCAAT boxes that presumably are binding sites for NF-Y (34). The results provide additional evidence that the two CCAAT boxes in the thymidine kinase promoter interact with proteins in vivo.

A summary of the in vivo footprinting results for the human TK promoter is shown in Fig. 6. From this genomic footprinting analysis, we have evidence for at least 14 different transcription factors. Among these footprints, we noticed a potential TATA box, two inverted CCAAT boxes and several Sp1-like binding sites. All of these sites were constitutively occupied by the factors as the cells proceeded from G0 into G1, S, and G2 phase.

FIG. 4. Genomic footprinting of the TK promoter in human fibroblasts. Lanes marked C, C + T; G + A, and G represent Maxam-Gilbert control sequences. In vitro DMS treated “naked” DNA (G lanes) is compared with DNA from fibroblasts that were serum-starved and treated in vivo with DMS at 0, 6, 12, 18, 24, or 30 h following serum stimulation. Lanes Div. Fib. and Div. HeLa, DNA from DMS-treated unsynchronized fibroblast and HeLa cell cultures, respectively. The footprints are indicated by boxes and brackets. A, sequences from the upper strand spanning nucleotides -7 to -137 were analyzed with primers tk-c1, -c2, and -c3. B, sequences from the lower strand spanning nucleotides +23 to -130 were analyzed with primers tk-a1, -a2, and -a3.
DISCUSSION

The Identity of the Protein-DNA Complexes at the Human TK Promoter—Several lines of evidence indicate that transcriptional regulation plays a key role in induction of the human TK gene in normal diploid cells following serum stimulation (45, 49, 50) and that the 5′-flanking sequences of the TK gene contribute significantly to its S phase-specific stimulation (23, 51). In this study, we examined cell cycle-specific binding of transcription factors at the promoter of the human TK gene. Fourteen putative protein-binding sites were identified in vivo by a genomic footprinting technique (for a summary see Fig. 6), suggesting that transcriptional regulation of the gene in a periodic fashion is probably accomplished through complex mechanisms. Ten of these sites correspond to well known regulatory elements, as deduced by sequence inspection and comparison with known factor-binding sites. They include a TATA rich element which resembles a TATA box, two inverted CCAAT boxes and a series of GC elements, probably binding sites for Sp1, further upstream. The presence of CCAAT boxes and Sp1 transcription factor binding sites is a common feature of many cell cycle-regulated genes (18).

The Role of E2F in TK Regulation—The core of the CCRU contains three E2F-like sites (Fig. 7) and has been shown to form minor S-phase-specific complexes containing p107, cyclin A, and p33 cdK2, in nuclear extracts isolated from growth-stimulated cells (27). Later, Chang et al. (42) showed that the activity of the human TK promoter is positively modulated by the steady-state levels of cyclin A and cyclin E in normal IMR-90 human fibroblasts and that this transcriptional activation is mediated via the CCRU region. Nevertheless, the TK-specific complexes identified in previous work showed very little affinity for an oligonucleotide containing a consensus E2F motif (27, 28, 30). In our hands, competition analysis using a synthetic oligonucleotide containing an authentic E2F-binding sequence together with antibody perturbation experiments showed instead the direct involvement of E2F-4 and p130 in TK promoter-binding complexes (data not shown). Although suggestive, these in vitro binding experiments do not necessarily prove that E2F is involved in binding in vivo.

The in vivo footprinting analysis shows that major protein contact points occur at one of the putative E2F sites where three Gs are protected from DMS modification in vivo. This site is in the middle of the sequence shown to be responsible for cell cycle-dependent TK promoter transcriptional activation. Site-directed mutagenesis experiments were able to further restrict the critical regulatory domain to a 14-bp region between −97 and −84 (30). This region includes two oppositely oriented overlapping E2F-like sites (sites 2 and 3 in Fig. 7) but not the
abutting E2F-like site 1, therefore excluding an involvement of site 1 in cell cycle regulation. Site 2/3 was shown to be occupied in vivo at all stages of the cell cycle (Fig. 4).

Since regulatory factors bind constitutively to their target sites, post-translational modifications such as phosphorylation and/or additional protein-protein interactions with other regulatory factors may be postulated as possible mechanisms for activating the gene at the G1/S border. The nature of such interactions that occur in vivo at the TK promoter cannot be analyzed directly with currently available methods.

Comparisons with Other E2F-regulated Promoters—Our previous in vivo and in vitro data, together with mutational analysis, showed that an inhibitory protein complex containing the heterodimer E2F-4/DP-1 and the pocket protein p130 binds at the -20 element of the human cdc2 promoter in serum-arrested fibroblasts (17). This complex is released at the G1/S transition in coincidence with cdc2 activation. Such a pattern of binding suggests that the E2F-4/p130 repressor, bound in proximity of the transcription start site, may interfere with the components of the basal transcription machinery and therefore may play a crucial role in preventing cdc2 expression at inappropriate phases of the cell cycle (17).

Table I shows that, independently of the mechanism(s) of regulation, all these promoters contain a GC-rich E2F binding motif and a TGG/A motif, designated as CDE and CHR, respectively. Table I shows that, independently of the mechanism(s) of regulation, all these promoters contain a GC-rich E2F binding motif and a TGG/A motif, designated as CDE and CHR, respectively.

| Gene          | Consensus sequence | Rep/Act | TSS box  | In vivo complexes | References |
|---------------|--------------------|---------|----------|-------------------|------------|
| CycA         | ATATCACTGGGC       | Rep     | -        | G0/G1             | (61)       |
| cdc2         | TTTAAGGTTACG       | Rep     | -        | G0/G1             | (17)       |
| cdc25        | GGCCCTCGGG         | Rep     | -        | G0/G1             | (55)       |
| B-Myb        | CACCTGGCCAGA       | Rep     | -        | G0/G1             | (12,19,52,53) |
| TK           | GACCTGGCCAGA       | Act     | +        | G0/G1/S/G2        | (27,30,61) |
| E2F-1        | GATTCCGGGTA         | Rep     | -        | N.D.              | (13,14)   |
| DNA Pol.α    | GTTTGGCCGGCG       | Act     | +        | N.D.              | (10)       |
| DHFR         | AATTCCGGCGA        | Act     | -        | G0/G1/S           | (64,66)   |
| Adeno E2a    | GTTTGGCCCA          | Act     | +        | N.D.              | (63,56)   |
| p107         | ATTTGGCCCA          | Act     | -        | N.D.              | (67)       |
| Hsorc1       | AACTGGCCAGA        | Rep     | -        | N.D.              | (69)       |
| H2A          | TTTTCCGGCGCAAGCTTGCGA | Act     | +        | N.D.              | (57)       |
| c-myc        | TTTTCCGGCGCAAGCTTGCGA | Rep     | -        | G0/G1/S           | (56,55)   |
by which the transcription factor E2F exerts its control over the target gene, acting either as a repressor or as an activator, all sequences analyzed shared a high degree of homology. These promoters contain both the CDE and the CHR elements, separated from each other by a stretch of 3–7 nucleotides (mostly AT-rich).

This invites a number of conclusions and speculations. First, the presence of the CDE and CHR elements is not an exclusive feature of genes which are under E2F-mediated negative control (as previously proposed in Zwicker and Müller (18)), but may be part of a more general mechanism of E2F-mediated regulation. The evidence that promoters of growth-regulated genes other than TK, such as the adenovirus E2A gene and the human H2A and c-myc genes, for which the involvement of E2F as a transcriptional activator is well documented (5, 56, 57), share identical CDE and CHR elements clearly supports this hypothesis (Table I). We recently demonstrated that a “high affinity” binding site for E2F at position −128, previously described as the main modulator of human cdc2 promoter activity (58), is in fact not responsible for the periodic transcriptional regulation of this gene, since no protein complexes have been found to bind in vivo throughout cell cycle progression (17). In light of these data, we analyzed the sequences encompassing this putative E2F site, and no CHR element was found, thus suggesting that the CHR element, together with the GTG-rich core region of the CDE, may be necessary for in vivo binding of E2F complexes. It is noted, however, that despite the strong sequence homologies, no significant in vivo DMS footprint was found at the CHR elements in the cdc2 promoter (17) and the TK promoter (this study), but was detected in the cyclin A gene (55).

It has been suggested that different members of the E2F family may be involved in its dual modulation of growth control, since members of the E2F family may have different affinities for slightly different E2F sites. This, apparently, seems not to be the case. Our mobility shift data show that E2F-4 binds to the cdc2 repressor element as well as to the transactivating element in the TK gene in G1/G0 early G1 (17) (data not shown). Nevertheless, we cannot rule out the possibility that single-nucleotide changes in the sequence may determine a subtle variation in the protein-binding affinities or complex composition during the cell cycle, thus allowing members of the E2F family or different higher order complexes to discriminate specific sequences.

The E2F-dependent repressor elements are all located in close proximity to the transcription initiation site (−50 to +20) in TATA-less promoters, suggesting that sterical interference with the basal transcription machinery may act as a common mechanism of repression. The only exception to this rule appears to be the dhfr gene, in which an activating element is located close to the transcription start site (59). A human histone H2A gene does have a TATA box between the activating E2F site and the transcription start site (57). At the onset of S phase, when all these genes are activated, the E2F-bound repressors are released and the transcription initiation complex can bind the DNA upstream of or near the start site. The mechanism for the release of the repressing E2F complexes is unknown. Release could involve Cdk-driven phosphorylation events or could be accomplished through components of the basal transcription apparatus. The lack of canonical TATA boxes in these E2F-repressed promoters, together with the finding that E2F can interact in vitro with TBP at early stages in the assembly of the preinitiation complex (60), may even suggest that the E2F-complexes are perhaps attracting the TBP-containing complex to the transcription start site, but E2F is then released immediately before the initiation of transcription at E2F-repressed promoters.

In other promoters, such as TK, where E2Fs are involved as activators, the binding sites are located in a position farther 5’ to the transcription start site. A consequence of this is perhaps the free interaction of positive modulators (cyclins/Cdkks) with the transcription machinery through upstream E2F binding sites. Another possibility is that activating E2F complexes require Sp1 binding sites in their close proximity (61, 62).

At this point, the versatility of the transcription factor E2F to act either as a repressor or an activator in different genes could probably be seen as the result of a DNA binding-dependant mechanism. Repressing elements show G1/G0 phase-specific binding in vivo while binding at activating elements is constitutive (Table I). In addition to that, the phosphorylation of pRb and other pocket proteins, the association of E2F complexes with Cdkks and cyclins, the phosphorylation of E2F-DP1 complexes, and interactions with neighboring cis-acting elements are probably also crucial events which contribute to the relief of E2F-mediated repression and/or the induction of E2F-mediated transactivation and provide extra fine-tuning for the precise activation of these genes at specific time points during the cell cycle.

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