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The differential binding of E2F and CDF repressor complexes contributes to the timing of cell cycle-regulated transcription

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ABSTRACT

B-*myb* and *cdc25C* exemplify different groups of genes whose transcription is consecutively up-regulated during the cell cycle. Both promoters are controlled by transcriptional repression via modules consisting of an E2F binding site (E2FBS) or the related CDE plus a contiguous CHR co-repressor element. We now show that the B-*myb* repressor module, which is derepressed early (mid G_1), is preferentially recognized by E2F–DP complexes and that a mutation selectively abolishing E2F binding impairs regulation. In contrast, the *cdc25C* repressor module, which is derepressed late (S/G_2), interacts selectively with CDE–CHR binding factor-1 (CDF-1). E2F binding, but not CDF-1 binding, requires specific nucleotides flanking the E2FBS/CDE core, while CDF-1 binding, but not E2F binding, depends on specific nucleotides in the CHR. Swapping these nucleotides between the two promoters profoundly changes protein binding patterns and alters expression kinetics. Thus predominant CDF-1 binding leads to derepression in late S, predominant E2F binding results in up-regulation in late G_1, while promoters binding both E2F and CDF-1 with high efficiency show intermediate kinetics. Our results support a model where the differential binding of E2F and CDF-1 repressor complexes contributes to the timing of promoter activity during the cell cycle.

INTRODUCTION

E2F is a heterodimeric transcription factor composed of members of the E2F and DP multigene families. Transcriptional activation by E2F is modulated during the cell cycle by pocket proteins of the pRb family. E2F is repressed in G_0 and early G_1, but during cell cycle progression both the DP/E2F moiety and the associated pocket proteins are hyperphosphorylated by G_1-specific cyclin-dependent kinases leading to dissociation of the inhibitory ternary complex. This dissociation generates transcriptionally active 'free E2F' and leads to activation of E2F-regulated genes. In recent years, however, it has become clear that the role of E2F is not exclusively activating. This was first demonstrated for the mouse B-*myb* gene. Mutation of the E2F binding site (E2FBS) in the B-*myb* promoter leads to a dramatically increased activity selectively in G_0 and consequently to a loss of cell cycle regulation. Other examples in this context are the *E2F*-1, *p107* and *orc*-1 promoters, where mutations of E2FBS also abrogate repression and cell cycle regulation. The identification of several genes that are repressed through E2FBS suggests that E2F-mediated transcriptional repression is a frequent mechanism of cell cycle-regulated transcription. For other promoters there is clear evidence for E2F-mediated transactivation. These genes include c-*myc*, *cyclin E* and *tk* (thymidine kinase). In these cases E2FBS mutations lead to a significant decrease in promoter activity, as would be predicted for a transcriptional activator. An important question is thus why structurally nearly identical E2FBS in different promoters act as either repressor or activator elements.

The analysis of genes that are expressed at later stages of the cell cycle provided the first hint regarding this question. *In vivo* footprinting and mutational analysis of the *cdc25C*, *cyclin A* and *cdc2* promoters, which are up-regulated in S phase, led to the discovery of a novel repressor element, the cell cycle-dependent element (CDE), which is specifically occupied when these promoters are not transcribed. These studies also led to the discovery of an additional element contiguous with the CDE, termed the cell cycle genes homology region (CHR). Mutation of either the CDE or the CHR in the *cdc25C*, *cdc2* or *cyclin A* promoters largely abolishes repression in G_0. Interestingly, the CDE is contacted in the major groove of the DNA while binding to the CHR occurs in the minor groove. In the acompanying paper by Liu *et al*. we show that the CDE–CHR module interacts with a novel E2F-unrelated factor termed CDF-1.

The discovery that the CHR cooperates with a CDE in repression of promoters and identification of CHR-like sequences adjacent to the E2FBS in the B-*myb* promoter, prompted detailed investigations into the mechanism of B-*myb* repression. These

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studies showed that the CHR-like region is indispensible for repression and acts as a co-repressor element together with the E2FBS. This region has been termed the B-*myb* CHR or DRS. In addition, genomic footprinting clearly showed a loss of E2F site occupation paralleling derepression of B-*myb* in mid G_1. These observations suggest that E2F–CHR sites regulate transcription of genes induced in late G_1 in a similar way to that by which CDE–CHR sites lead to derepression of genes in S or G_2. In addition, these findings suggest that repressing E2F sites differ from activating E2F sites by the presence of a contiguous CHR co-repressor element.

However, a number of issues remains unresolved at present. Thus the CDE is identical to E2FBS core sequences, such as those in the B-*myb* promoter (GGCGG), but it remains elusive what determines the distinction of an E2FBS from a CDE. Likewise, it is unknown what the functional differences between E2F and the CDE–CHR binding protein CDF-1 are, in particular with respect to the kinetics of promoter regulation during cell cycle progression. In the present study we have analyzed the molecular basis for the differential binding of CDF-1 and E2F to specific promoters and established correlations between the binding of CDF-1 and/or E2F complexes and the timing of promoter activity during the cell cycle.

MATERIALS AND METHODS

Cell culture, DNA transfection and luciferase assays

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. HeLa cells were grown in DMEM plus 5% newborn calf serum. NIH 3T3 cells were transfected by the DEAE–dextran technique. For synchronization in G_0 cells were maintained in serum-free medium for 60 h after transfection and restimulated with 10% FCS at the times indicated in the respective figures. Determination of luciferase activities and standardization of results using SV40 promoter-driven reporter constructs were performed as published.

Sequence analysis and luciferase constructs

The *cdc25C* and B-*myb* promoter-driven luciferase constructs have been described elsewhere. Mutations were introduced by PCR strategies as previously described. All PCR-amplified fragments were verified by DNA sequencing using the dideoxynucleotide chain termination method using Sequenase 2.0 (US Biochemical) or Tth polymerase (Pharmacia).

Partial purification of CDF-1

Nuclear extracts were prepared from HeLa suspension cultures in high salt extraction buffer in the presence of the protease inhibitors leupeptin (50 ng/ml), pepstatin A (5 µg/ml) and aprotinin (80 ng/ml). A biotinylated oligonucleotide containing two tandem *cdc25C* CDE–CHR motifs was coupled to streptavidin–agarose and used for affinity chromatography as described under the same conditions as for EMSA (see above), except that salmon sperm DNA instead of poly(dA:dT) was used as the non-specific competitor. Elution was performed by stepwise increasing the KCl concentration to 1 M. CDF-1 was eluted at a salt concentration of 300–400 mM.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously. When partially purified CDF-1 was used the EMSA was carried out in the absence of sodium deoxycholate and NP-40. Details of the EMSA procedure are indicated in the accompanying manuscript by Liu *et al*. The following double-stranded probes were used: cdc25C-wt, 5′-ACTGGGCT**GGCGG**AAGG*TTTGAA*TGGTCAA (CDE bold, CHR italic); T1, T4, T7 (also referred to as *cdc25C*-mCDE), A8 and C9 are mutated at positions –19, –16, –13, –12 and –11 respectively as described (24); cdc25C –10/–7, 5′-ACTGGGCTGGCGGA*cttg*-TTGAATGGTCAA; cdc25C –6/–3 (also referred to as *cdc25C*-mCHR), 5′-ACTGGGCTGGCGGAAGGT*ggtc*ATGGTCAA; cdc25C –1/+2, 5′-ACTGGGCTGGCGGAAGGTTTGAA*ggt*T-CAA; cdc25C-2, 5′-ACTGGGCTGGCGGAAGGTTTGA*c*TGG-TCAA. The sequences of all other oligonucleotides, including B-*myb*, have been described elsewhere or are indicated in Figure 1. The random oligonucleotide contains an irrelevant sequence. The following antibodies were used: E2F-1 (Santa Cruz SC-251X), E2F-3 (Santa Cruz SC-879X), E2F-4 (Santa Cruz SC-512X; also kindly provided by R.Bernards, Amsterdam) and DP-1 (obtained from N.La Thangue).

RESULTS

Identification of nucleotides determining preferential E2F or CDF-1 binding

We first sought to investigate the unresolved issue of what discriminates an E2F repressor site from a CDE–CHR module. These analyses were complicated by the fact that DP–E2F and CDF-1 complexes show very similar electrophoretic mobilities on EMSA. We therefore fractionated HeLa nuclear extract by DNA affinity chromatography using a 20 bp *cdc25C* CDE–CHR sequence (see Materials and Methods for details). This procedure yielded partially purified CDF-1 showing very similar binding properties to the CDF-1 in crude extracts and gave a complete separation of CDF-1 from the E2F binding activity (data not shown). This partially purified fraction was used for all analyses of CDF-1 binding, while HeLa nuclear extract was used for the analysis of E2F complexes. In the latter assays a *cdc25C* CDE–CHR competitor oligonucleotide was included in the binding reactions to prevent formation of radiolabeled CDF-1–DNA complexes.

To address the question what determines binding of DP–E2F and CDF-1 we swapped specific nucleotides between the B-*myb* and *cdc25C* promoters in five specific regions where the repressor modules differ from each other (denoted 1–5 at the top of Fig. 1). The corresponding sequences were first tested for E2F binding (i.e. binding of DP1–E2F-1, -3 and -4 in HeLa nuclear extract) and interaction with partially purified CDF-1. This study yielded two clear results.

(i) The nucleotides flanking the CDE or the core of the E2FBS (regions 1 and 2) play an important role in E2F binding. In contrast, the same positions do not noticeably influence CDF-1 binding. While the nucleotides in region 1 (CT in B-*myb*) mainly influence the maximum binding of DP1–E2F-4 (B-C1 in Figs 1 and 2A), the G residue in region 2 is crucial for interaction with all E2F complexes (B-C1,2 and B-C2 in Fig. 1 and B-C1,2 in Fig. 2A). In agreement with this conclusion, the introduction of B-*myb* regions 1 and 2, but not region 1 alone, confers on the *cdc25C* CDE the ability to interact with DP1–E2F-1, -3 and -4 complexes with high efficiency (C-B1,2 in Figs 1 and 3B). In

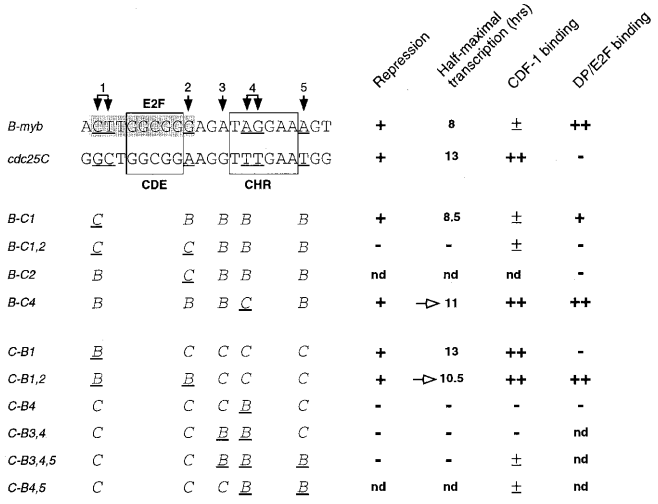

Figure 1. Effects of specific nucleotide exchanges between the B-*myb* E2FBS–CHR module and the *cdc25C* CDE–CHR motif on cell cycle regulation and DNA binding of E2F and CDF-1 complexes. The B-*myb* and *cdc25C* repressor modules are shown at the top. Five positions where the sequences differ from each other were designated regions 1–5. Each of the mutants indicated below harbors specific exchanges between the two promoters in a B-*myb* (upper block) or *cdc25C* (lower block) promoter background. B and C indicate whether the particular mutant contains *cdc25C* (C) or B-*myb* (B) nucleotides in regions 1–5 (e.g. B-C1 is a B-*myb* sequence containing the *cdc25C* nucleotides in region 1). Cell cycle regulation was measured first by comparing the activity of wild-type and mutant constructs in quiescent NIH 3T3 cells. The column designated Repression summarizes the results of this analysis. +, 1.4- to 2.7-fold cell cycle regulation (RLUs growing/RLUs G_0); –, 9- to 20-fold cell cycle regulation (RLUs growing/RLUs G_0). The functional promoter constructs were then analyzed for timing of cell cycle regulation in serum-stimulated NIH 3T3 cells (see Fig. 4 for details) and the times of half-maximal activities were determined. Hollow arrows indicate kinetics that clearly differ from both B-*myb* and *cdc25C* wild-type promoters. CDF-1 and E2F binding data were obtained by EMSA in the experiments shown in Figures 2 and 3.

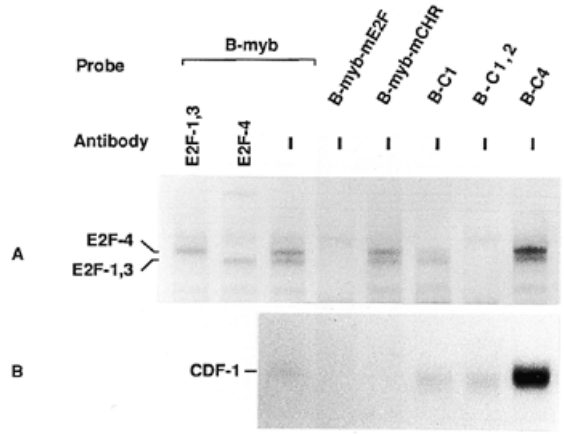

Figure 2. Effects of specific nucleotide changes on DP–E2F (**A**) and CDF-1 (**B**) complex formation with wild-type and mutated B-*myb* E2FBS–CHR probes using HeLa cell nuclear extract (A) or partially purified CDF-1 (B). In (A) the *cdc25C* oligonucleotide was included as a competitor to be able to identify the E2F complexes. The wild-type *cdc25C* oligonucleotide abolishes formation of radiolabeled CDF-1 complexes so that the E2F complexes, which have a very similar mobility, become clearly discernible. See Figure 1 for nomenclature.

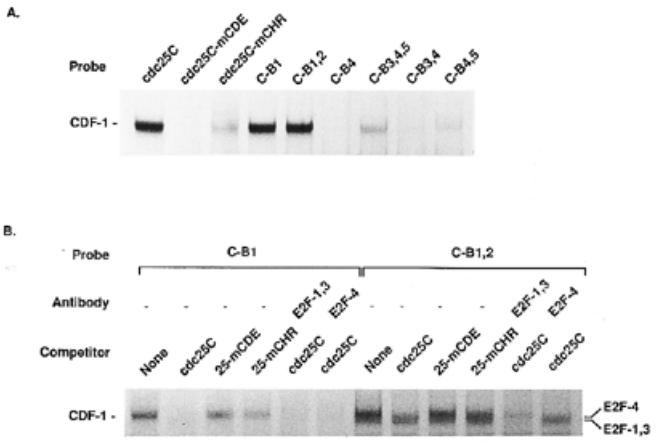

Figure 3. Effects of specific nucleotide changes on CDF (**A**) and DP–E2F (**B**) complex formation with wild-type and mutated *cdc25C* CDE–CHR probes using partially purified CDF-1 (A) or HeLa cell nuclear extract (B). In some binding reactions in (B) antibodies were included to identify specific E2F complexes. Competitors were added as indicated in (B) to show the specificity of complex formation and to be able to discriminate between CDF-1 and E2F complexes, which have a very similar mobility. See Figure 1 for nomenclature.

contrast, none of these nucleotide changes around the E2FBS core or the CDE affected binding of CDF-1 (B-C1 and B-C1,2 in Figs 1 and 2B, B-C2 in Fig. 1 and C-B1 and C-B1,2 in Figs 1 and 3A).

(ii) The converse was true for CDF-1 binding: the structure of the CHR had a strong impact on CDF-1 binding while not influencing E2F binding and, in this respect, region 4 was the crucial one. Thus exchange of 2 nt in this region between *cdc25C* and B-*myb* led to a strong increase in CDF-1 binding to the B-*myb* promoter (B-C4 in Figs 1 and 2B), while the converse exchange destroyed binding of CDF-1 to the *cdc25C* promoter (C-B4 in Figs 1 and 3A). In contrast, the changes in the CHR in region 4 did not affect binding of E2F complexes. Since it was formally possible that the B-*myb* CHR extended beyond the borders determined for the *cdc25C* CHR and the two promoters differ in these positions (regions 3 and 5 in Fig. 1), we could not exclude that C-B4 did not interact with CDF-1 due to an incomplete B-*myb* CHR. We therefore also introduced the B-*myb* nucleotides found in regions 3 and 5 into the *cdc25C* sequence in addition to the change in region 4 (C-B3,4, C-B3,4,5 and C-B4,5 in Figs 1 and 3A). However, these additional alterations could restore CDF-1 binding only to a marginal extent, confirming that the B-*myb* CHR and *cdc25C* CHR sequences are not equivalent with respect to interacting proteins.

Expression kinetics correlate with differential binding of E2F and CDF-1

We next analyzed how the differential interaction of E2F and CDF-1 complexes with the B-*myb* and *cdc25C* promoters would affect cell cycle-regulated transcriptional repression and the timing of regulation. The same sequences tested in Figure 1 for binding of E2F and CDF-1 were introduced into B-*myb* and *cdc25C* promoter–luciferase constructs and tested for activity in serum-stimulated NIH 3T3 cells that had been synchronized in

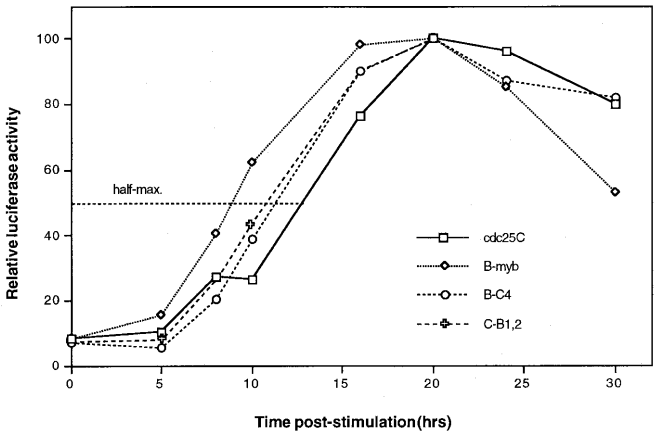

Figure 4. Effects on cell cycle kinetics of specific nucleotide changes in the B-*myb* and *cdc25C* promoters leading to binding of both E2F and CDF-1 to the same site. NIH 3T3 cells were transiently transfected with the indicated constructs (see Fig. 1 for nomenclature), synchronized in G_0 by serum deprivation and stimulated for the indicated times. The data is based on 12 different experiments, except for the C-B1,2 graph, which is based on four experiments. Data were normalized to 100 at 20 h for each construct in order to facilitate a comparison of the half-maximal expression values.

Figure 5. Sequence requirements for binding of and repression by E2F and CDF-1, based on the data obtained in the present and previous studies. Data are based on the B-*myb* (E2F) and *cdc25C* (CDF-1) promoter sequences.

G_0. The data in Figure 1 show that abrogation of E2F binding to the B-*myb* promoter in the presence of wild-type-like CDF-1 binding impairs repression in G_0 (see B-C1,2). This observation strongly suggests that E2F rather than CDF-1 complexes are responsible for cell cycle-regulated transcription of the B-*myb* gene, which is in agreement with the relatively low affinity of CDF-1 for the B-*myb* promoter. In contrast, mutations in the *cdc25C* CDE which abrogate CDF-1 binding also impair cell cycle regulation (see accompanying paper by Liu *et al.*). Likewise, replacement of the *cdc25C* CHR with that of B-*myb* abolishes CDF-1 binding as well as repression in G_0 (C-B4, C-B3,4 and C-B3,4,5 in Fig. 1). Interestingly, the converse construct harboring a *cdc25C* CHR in a B-*myb* promoter background (B-C4) showed intermediate cell cycle kinetics, i.e. a delay in derepression of transcription relative to wild-type B-*myb* by 3 h (Figs 1 and 4). This construct binds CDF-1 with increased efficiency, suggesting that the ability to interact with both E2F and CDF-1 complexes leads to derepression after S phase entry, i.e. later than B-*myb* but prior to *cdc25C*. This conclusion is supported by the very similar cell cycle kinetics observed with C-B1,2 (Figs 1 and 4), where changes to the CDE endowed the *cdc25C* promoter with the ability to interact with both E2F and CDF-1 with high efficiency (Figs 1 and 2B). These findings clearly indicate that differential binding of E2F and CDF-1 complexes determines the cell cycle kinetics of the promoters tested in the present study.

DISCUSSION

Transcriptional repression plays a crucial role in the regulation of cell cycle genes. A major factor implicated in cell cycle-regulated repression is E2F, as shown for the *E2F*-1, *orc*-1 and B-*myb* promoters. The mechanism of B-*myb* gene repression appears, however, to be unique in view of two different observations. First, it requires a second element located directly downstream of E2FBS. Second, occupation of the B-*myb* E2FBS *in vivo* is found specifically during phases of repression. These observations are reminiscent of those made with promoters which are periodically repressed through CDE–CHR modules, such as *cdc25C*, *cdc2* and *cyclin A*. Despite these similarities, both types of promoters are regulated by distinct factors. Thus we show in the present study that the B-*myb* gene is repressed through E2F complexes, while the *cdc25C* promoter is repressed by a novel activity identified in the accompanying paper by Liu *et al.*, CDF-1. Based on these results we have addressed the question what distinguishes a repressing E2FBS (as in B-*myb*) from a CDE (as in *cdc25C*) with respect to both their recognition by specific factors and their function in specific phases of the cell cycle. Our data strongly suggest that differential binding of E2F and CDF-1 contributes to phase-specific repression of genes during the cell cycle.

The fact that the B-*myb* promoter E2FBS binds CDF-1 only relatively weakly does not necessarily mean that CDF-1 is not involved in B-*myb* repression. We therefore sought to identify those nucleotides in the E2FBS of B-*myb* and the CDE of *cdc25C* that are responsible for discriminating between E2F and CDF-1 binding. The data presented in Figures 1–3 and summarized in Figure 5 clearly show that these are the nucleotides directly adjacent to the E2FBS/CDE core GGCGG. Thus the 3 nt upstream (CTT in B-*myb*) and 1 nt downstream (G in B-*myb*) are crucial for E2F binding but not for CDF-1 binding. Interestingly, this additional G residue is also protected in the B-*myb* promoter *in vivo*. Based on these findings it was possible to assay the function of a mutant B-*myb* promoter with strongly reduced E2F binding but normal (i.e. weak) CDF-1 interaction and to show that repression of this construct is impaired. This data shows that interaction with E2F is crucial and that the weak binding of CDF-1 is insufficient to confer any cell cycle regulation on the B-*myb* promoter. Since E2F can bind to the B-*myb* promoter in a B-*myb* CHR-independent fashion even though the B-*myb* CHR is crucial for repression (see Fig. 5), it is unclear at present what the function of the B-*myb* CHR interacting protein(s) is. To address this question it will be necessary to identify such a factor(s) and to determine its (their) effect on other proteins interacting with the B-*myb* promoter, including E2F, transcriptional activators and regulatory molecules controlled by the cell cycle, such as pocket proteins.

The situation is very different for the CDE–CHR-repressed *cdc25C* promoter. In this case no binding of E2F is found and strong interaction with CDF-1 is critically dependent on the CHR (Figs 1 and 5). Thus the E2F binding site is larger (i.e. at least 9 nt) than the 5 nt CDE but does not include the CHR, while the CDF-1

binding site consists of the 5 nt CDE and the contiguous 6 nt CHR (see Fig. 5 and accompanying paper by Liu *et al.*; 34). It was therefore possible to create promoters which possess the ability to interact with both E2F and CDF-1 with high efficiency (Figs 1–4), either by changing the B-*myb* CHR to a *cdc25*C CHR (B-C4) or by changing the *cdc25C* CDE flanking nucleotides to their B-*myb* counterparts (C-B1,2). Interestingly, these promoters showed novel properties with respect to timing of derepression during the cell cycle, in that half-maximal activity was observed later than with B-*myb* but prior to *cdc25C*. These observations show that the differential binding of E2F and CDF-1 contribute to the timing of regulation. In agreement with this observation, we found that a B-*myb* promoter mutant showing preferential and strong CDF-1 binding (B-C1,3,4) shows *cdc25C*-like expression kinetics.

Our results show that CDF-1 is able to repress transcription in the context of the B-*myb* promoter, while E2F–DP seems to be able to exert a similar effect on the *cdc25C* promoter. Therefore, both proteins are able to repress the activity of transcription factors that are bound constitutively to the upstream promoter region of B-*myb* and *cdc25C*. The interchangeability of the repressor elements is striking because it indicates that E2F–DP and CDF-1 employ very similar mechanisms in repressing the activity of upstream transcription factors. The transcription factors primarily responsible for activation of *cdc25C* are the Gln-rich activators NF-Y and Sp1, while the B-*myb* promoter appears to be activated by Sp1. In that context it is interesting to note that many other cell cycle-regulated promoters, like *cdc2*, *cyclin A* and *E2F*-1, contain a very similar composition of upstream activators.

There are now at least four different mechanisms by which genes are regulated during the cell cycle through E2FBSs or CDE–CHR elements. These include: (i) activation by E2F, as shown for *cyclin E* and *histone H2A*; (ii) repression by E2F–pocket protein complexes through two inverted and overlapping E2FBSs in the absence of any recognizable CHR, as for example in the *p107* and *orc*-1 promoters; (iii) repression through the cooperative action of E2F and a factor binding to a contiguous CHR-like element, as in the B-*myb* promoter; (iv) repression by CDF-1 via cooperative binding to a CDE and a CHR, as in *cdc25C*, *cdc2* and *cyclin A* in the regulation of genes that become active at later stages. In this context it may be relevant that the CDEs in the *cyclin A* and *cdc2* promoters have been shown to interact with both CDF-1 (accompanying paper by Liu *et al.*) and E2F complexes, which might offer an explanation for their up-regulation prior to *cdc25C*. At present it is unclear how E2F can influence the timing of CDE-mediated repression. It is possible that E2F complexes, whose abundance increases after passing the restriction point, compete with CDF-1 for DNA binding. In this way E2F may function as an 'anti-repressor' and might also actively contribute to transcriptional activation. The present study provides the basis for addressing these important functional questions in future investigations.

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