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CDI-1-mediated repression of cell cycle genes targets a specific subset of transactivators

Jörk Zwicker+, Frances C. Lucibello, Valrie Jérôme, Sabine Brüsselbach and Rolf Müller*

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-UniversitSt Marburg, Emil-Mannkopff-Strasse 2, D-35033 Marburg, Germany

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ABSTRACT

The *cdc25C*, *cyclin A* and *cdc2* genes are regulated during the cell cycle through two contiguous repressor binding sites, the CDE and CHR, located in the region of transcription initiation and interacting with a factor termed CDF-1. The target of this repression seems to be transcriptional activation of these promoters by transcription factors bound upstream. The majority of these factors falls into the class of glutamine-rich activators, suggesting that CDF-1-mediated repression might be activation domain specific. In the present study we have used chimeric promoter constructs to demonstrate that the *cdc25C* UAS, but not the core promoter, is crucial for repression. In addition, we show that only specific transcription factors and activation domains are responsive to CDE–CHR-mediated cell cycle regulation. These observations clearly indicate that CDF-1 interferes with activation of transcription by a specific subset of transactivators. The repressible activation domains belong to the same class of glutamine-rich activators, pointing to specific interactions of CDF-1 with components of the transcription machinery. In agreement with this conclusion we find that a simple inversion of the CDE–CHR module completely abrogates cell cycle-regulated repression.

INTRODUCTION

We have chosen the human *cdc25C* gene (1) as a model system to investigate regulation of S/G_2-specific transcription in mammalian cells (2–5). To elucidate the mechanisms involved in cell cycle-regulated transcription of the *cdc25C* gene the human promoter was cloned and a comprehensive structure–function analysis was performed (3). Transient expression studies and *in vivo* footprinting studies led to the identification of two contiguous regulatory elements, termed the cell cycle-dependent element (CDE) and the cell cycle genes homology region (CHR) (3,4,6). These elements are located near the transcription initiation sites and play a key role in periodic transcription of the *cdc25C* gene. The CDE and CHR are bound by a transcriptional repressor in G_0/G_1 which is released in S/G_2 (3,4). The CDE–CHR interacting factor has been identified and termed CDF-1 (see accompanying paper by Liu *et al.*). The CDE apparently does not interfere with basal transcription from the core promoter (3). Its function is dependent on a stretch of upstream sequences that is needed for transcriptional activation (UAS). This led to the hypothesis that CDF-1 may function by regulating the activity of upstream activators in a cell cycle-dependent fashion (3,5). This conclusion is supported by the observation that the proteins interacting with the *cdc25C* UAS do not only bind constitutively *in vivo*, but in a heterologous context also activate transcription in a way that is not significantly influenced by the cell cycle (5).

The major transactivator of the *cdc25C* UAS is the transcription factor CBF/NF-Y, which binds to three sites 5′ of the CDE (5). A second important transactivator is Sp1 (or other members of the Sp family), which interacts with two sites further upstream (5). Interestingly, the major activation domains in both Sp1 (7) and NF-Y (8,9) are glutamine rich and both factors are therefore likely to contact a similar set of basal transcription factors, TAFs or other components of the preinitiation complex (10). It cannot, however, be ruled out at present that CDF-1 simply functions by preventing protein contacts through steric hindrance (11). It is therefore of obvious importance to consider the question as to whether this repression mechanism is restricted to a certain class(es) of activation domains (12) and thus activator–basal complex contacts (10). In support of such a hypothesis is the observation that the heterologous SV40 enhancer, which is bound by multiple transactivating factors belonging to different classes (13), is less efficiently repressed than the *cdc25C* UAS (3).

The relevance of this question is stressed by the fact that the promoters of various other cell cycle genes, such as *cdc2* and *cyclin A*, are also regulated through CDE–CHR elements and harbor multiple Sp1 and NF-Y sites in the UASs (4,14). In addition, cell cycle genes repressed by transcription factor E2F also show a conspicuous preference for Sp1 and NF-Y sites 5′ of the E2F sites (14). It is therefore likely that the molecular basis for E2F- and CDE-mediated negative regulation is very similar and that repression of glutamine-rich activators like Sp1 and

*To whom correspondence should be addressed. Tel: +49 6421 286236; Fax: +49 6421 288923; Email: mueller@imt.uni-marburg.de

+Present address: Howard Hughes Medical Institute, Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, CA 94720-3204, USA

The authors wish it to be known that, in their opinion, the first two authors should be considered as joint First Authors

NF-Y is a common mechanism of cell cycle-regulated transcription.

In the present study we have addressed the putative activator specificity of CDF-1-mediated repression in detail. To this end we have investigated how an exchange of the *cdc25C* UAS or the core promoter with heterologous sequences would affect CDE–CHR-mediated repression. In addition, we have analyzed the responsiveness of specific transcription factors and activation domains to CDE–CHR-mediated cell cycle regulation. The results of these analyses are compatible with the conclusion that CDF-1 interferes with activation of transcription by a specific subset of transactivators, which all belong to the class of glutamine-rich activators. We also find that a simple inversion of the CDE–CHR module completely abrogates cell cycle-regulated repression. Taken together, these observations point to specific interactions of CDF-1 with components of the transcription machinery.

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. Cells were transfected by the DEAE–dextran technique. For synchronization in G_0 cells were maintained in serum-free medium for 2 days after transfection. Determination of luciferase activities were performed as published. Mel-ab cells (15) (provided by Prof. I.Hart, London, UK) were grown in DMEM plus 10% FCS, 200 nM TPA and 10 mM cholera toxin. C2C12 cells (16) (ATCC CRL-1772; obtained from Prof. H.H.Arnold, Braunschweig, Germany) were grown in DMEM plus 20% FCS. Synchronization of cells in G_0/G_1 was followed by FACS analysis as described (17).

Plasmid constructs

The *cdc25C* promoter–luciferase constructs C290, C75, C33 and C20 as well the CDE mutants R1 and T7 (referred to as mCDE in this study) and the CHR mutant –6/–3 (referred to as mCHR in this study) have been described elsewhere (3–5). Other constructs were generated by cloning of synthetic oligonucleotides with appropriate terminal overhangs or by PCR strategies as previously described (4,5,18). The following oligonucleotides were used (as five copies) for cloning of reporter constructs containing multiple transcription factor binding sites: NF-Y (Ea-Y, MHC class II promoter; 19), 5′-ATTTTTCTGATTGGTTAA;NFκ-B; mouse κ light chain enhancer (20), 5′-AGAGGGGACTTTCCGAGA; NF-1/CTF (high affinity binding site from adenovirus origin of replication; 21), 5′-TTTTGGCTACAAGCCAATA; Sp1, 5′-ATGGGGCGGAGA; (7); Gal4, CGGAGTACTGTCCTCCG (22). The oligonucleotides were sythesized with *Bam*HI and *Bgl*II termini and cloned into the *Bam*HI-digested *cdc25C* promoter construct C20 (3). The Gal4 expression vectors are based on pGal(1–147) (23) (GAYA plasmids, see Table 1) or pCG (22) (all other constructs). Chimeric promoters were generated by fusing the following enhancers or UASs to the *cdc25C* construct C20 (3): human troponin C promoter from –98 to +23 (24); human *myf*-4/myogenin promoter from –210 to +54 (25); human tyrosinase enhancer (–2.0 to –1.8 kb, *Eco*RI–*Nco*I fragment) (26); mouse *TRP*-1 promoter from –332 to –23 (27); SV40 promoter/enhancer from –281 to –45 (genomic region 273–36) (13); CMV enhancer (292–695 region from pcDNA3; Invitrogen). In the case of SV40 and CMV enhancers fusions were also made with the *cdc25C* constructs C33, C51 and C20/+30 (3). For inversion of the CDE–CHR in the *cdc25C* promoter the sequence 5′-GGGCTGGCGGAAGGTTTGAAT was changed to 5′-GTTCAAACCTTGCCT. Other constructs were cloned using PCR-generated promoter fragments as indicated in Results and the figures. 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).

Table 1. Gal4 activation domain vectors used in the experiments in Figure 4

Type of activation domain	Name of plasmid	Description	Origin
Gln-rich	GAL4-N/Oct	Oct-2 (amino acids 3–154)	T.Wirth
	pSCTEV GAL4 Sp1:Q2	Sp1 (amino acids 340–485)	W.Schaffner
	GAYA-6	NFY-A (amino acids 1–132 D26–53)	R.Mantovani
Ser/Thr/Gln-rich	GAYA-5	NFY-A (amino acids 1–233 D26–53)	R.Mantovani
	GAYA-11	NFY-A (amino acids 1–233)	R.Mantovani
Ser/Thr-rich	pSCTEVGAL4 ITF2:ST	ITF2 (amino acids 2–452)	W. Schaffner
Acidic	pCG GAL4 VP16	VP16 (amino acids 413–490)	R.Tjian
	pGAL4 Myc	c-Myc (amino acids 1–262)	M.Eilers
Pro-rich	pCG GAL4 CTF	CTF (amino acids 399–499)	W.Herr
Empty vector	pCG (1–94)	–	W. Herr
	pGal4 (1–147)	–	R. Mantovani

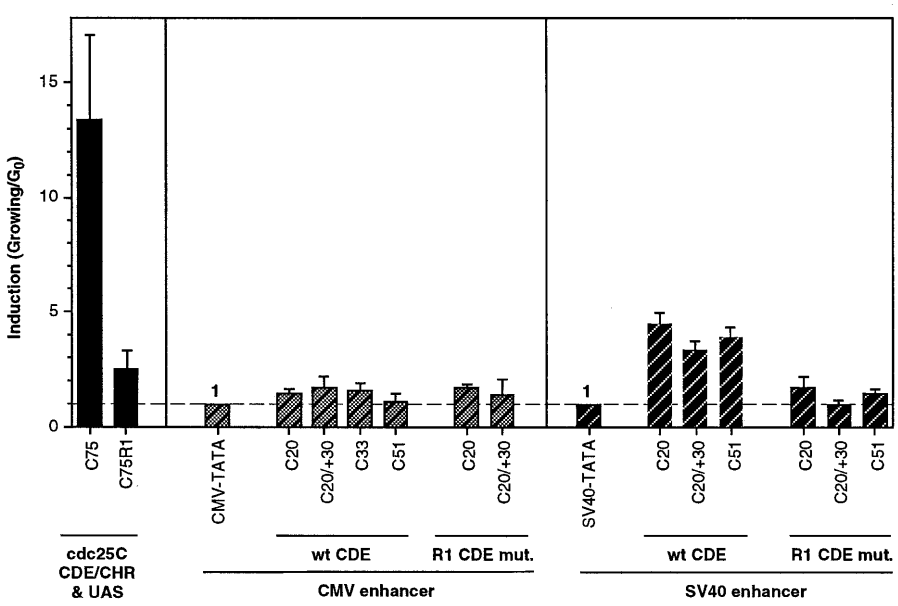


Figure 1. The SV40 enhancer, but not the CMV enhancer, is partially repressed in G_0 cells through the CDE–CHR in the context of the *cdc25C* core promoter. SV40 TATA and CMV TATA, natural SV40 and CMV regulatory sequences containing the viral early promoter/enhancer region, TATA box and transcription start site. Chimeric SV40 and CMV fusion constructs consist of the SV40 early promoter/enhancer region or the CMV enhancer linked to a minimal *cdc25C* promoter fragment harboring either a wild-type (wt) or mutated (R1) CDE and the core promoter. C20, *cdc25C* sequence from –20 to +121; C20/+30, –20 to +30; C33, –33 to +121; C51, –51 to +121. The C75 (wild-type) and C75R1 (mutated CDE) *cdc25C* promoter constructs (–75 to +121) were included for comparison. All constructs were tested in quiescent (G_0) and growing cells. Data are represented as the ratio of luciferase activity in growing versus G_0 cells, normalized to 1 for SV40 TATA (C75 and SV40 fusion constructs) or CMV TATA (chimeric CMV constructs). Values are given as averages ± SD calculated from two to four independent sets of data.

RESULTS

The *cdc25C* CDE–CHR module fails to repress heterologous enhancers

We first addressed the question whether CDE–CHR-mediated repression might be dependent on a specific UAS. For this purpose the *cdc25C* UAS was exchanged with various heterologous enhancer sequences. Since it could not be excluded that the spacing between activators and repressor might be important, the heterologous sequences were fused with various *cdc25C* promoter fragments starting at –51, –33 or –20 and extending to +121. In addition, we used a fragment lacking most of the downstream sequence, extending to +30. All fragments were used in the wild-type form and, as controls, with mutated CDEs. The *cdc25C* promoter fragments were fused with both the cytomegalovirus (CMV) enhancer and the simian virus 40 (SV40) early enhancer/promoter regions and tested for cell cycle-regulated repression in transient luciferase assays in NIH 3T3 cells. As shown in Figure 1, the chimeric CMV constructs did not show any significant cell cycle regulation, in contrast to the chimeric SV40 constructs. In the latter case cell cycle regulation was, however, only partially restored and ~3-fold below the induction value seen with the natural *cdc25C* promoter construct C290.

We also used the enhancer sequences from a number of other promoters in similar experiments (data not shown). The human tyrosinase enhancer (26) and tyrosinase-related protein-1 (*TRP*-1) UAS (27) was fused to a *cdc25C* promoter fragment (–20 to +121). The resulting chimeric constructs were tested in the melanocytic cell line Mel-ab (15), which can be synchronized in G_0 by serum deprivation. Analogous constructs were generated with the human *myf*-4/myogenin (25) and troponin C (24) UASs and tested in the myoblast cell line C2C12 (16). These cells were arrested in G_1 by exposure to 5% horse serum instead of 20% FCS (25). With none of the constructs were we able to observe any cell cycle regulation. These observations clearly suggest that efficient cell cycle-regulated repression is observed only with specific activating sequences, such as the *cdc25C* UAS.

The CDE–CHR module represses the *cdc25C* UAS in the context of heterologous core promoters

We next asked the question whether the core promoter might be of similar importance as the UAS. We use the term ‘core promoter’ for the transcription initiation region of the *cdc25C* gene. This region, which shows basal promoter activity, extends from approximately +1 to +50 (see accompanying paper by Körner *et al.*). To address this issue we fused a *cdc25C* promoter fragment (–290 to +2) harboring the UAS and the CDE–CHR, but lacking the core promoter, to the core promoters of the human terminal deoxynucleotidyl transferase (TdT) (28) and SV40 early genes (13). As can be seen in Figure 2, these chimeric promoters showed a similar cell cycle regulation as the wild-type promoter C290, indicating that CDE–CHR-mediated repression is not core promoter specific. In the subsequent experiments we therefore directed our attention to the observed activator specificity.

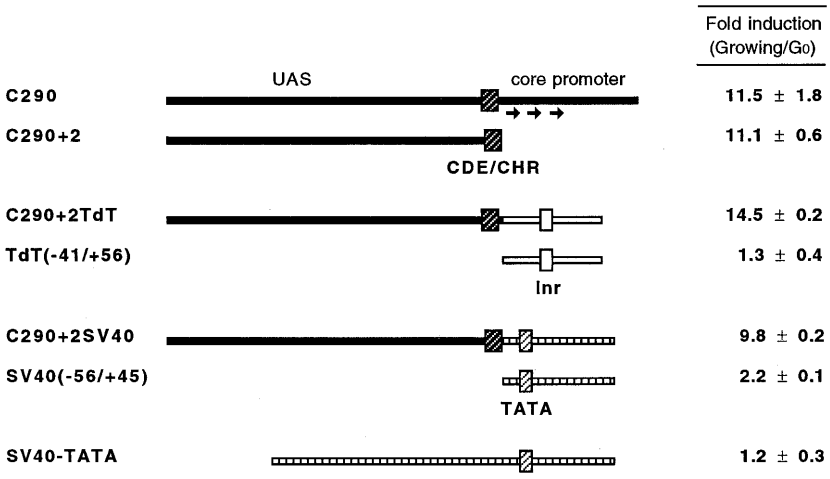
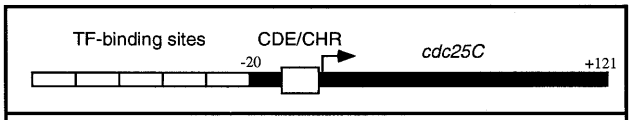


Figure 2. Repression through the CDE–CHR is functional with heterologous core promoters. The *cdc25C* UAS plus the CDE–CHR module (–121 to +2) were fused to the core promoters of the human terminal deoxynucleotidyl transferase gene (*TdT*) or the SV40 early region. The *cdc25C*, *TdT* and SV40 fragments alone, as well as the C290 *cdc25C* promoter construct (–290 to +121) and the SV40 TATA construct (see legend to Fig. 1) were included for comparison. Black bars, *cdc25C* sequences; white bars, *TdT* sequences; hatched bars, SV40 sequences. Data are represented as the ratio of luciferase activity in growing versus G_0 cells. Values are given as averages ± SD calculated from three independent experiments.

The *cdc25C* CDE–CHR module represses activation by specific transcription factors

The observations described above suggest that CDE–CHR-mediated repression might work only in the context of specific activators. To address this question more directly we sought to investigate the function of the CDE–CHR module in the context of promoters which interact with only one transcription factor (family). To this end we constructed promoters where five binding sites for NF-Y, NFκ-B, NF-I or Sp1 fused to the *cdc25C* minimal promoter construct C20 (–20 to +121). To be able to distinguish cell cycle effects exerted through the CDE–CHR from those due to other regulatory effects on the transactivators all constructs were generated with both a wild-type CDE and a mutated CDE. The constructs were tested in both growing and quiescent NIH 3T3 cells (Fig. 3). All four mutant constructs lacked any significant cell cycle regulation, as did the wild-type ones containing NFκ-B or NF-I binding sites. In contrast, CDE–CHR-dependent cell cycle-regulated transcription was seen with NF-Y sites (3-fold) and, to a lesser extent, with Sp1 sites (2.2-fold). Since NF-Y and Sp1 are the transactivators of the *cdc25C* promoter (5), these observations may explain the observed requirement for a specific UAS. The fact that Sp1 is subject to cell cycle regulation via the CDE–CHR module may also offer an explanation for the observation that the SV40 promoter, which contains six Sp1 sites (13), was the only one which could replace the *cdc25C* UAS without totally obliterating cell cycle-regulated repression (Fig. 1).

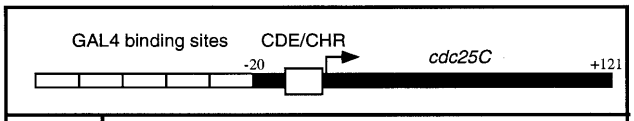


Binding site	RLUs Growing / RLUs G_0		Cell cycle regulation
	Intact CDE/CHR	Mutated CDE/CHR	
NF-Y	5.8 ± 1.1	1.9 ± 0.6	**3.0**
NFκ-B	1.5 ± 0.4	1.4 ± 0.5	**1.1**
NF-I	1.9 ± 0.3	1.7 ± 0.1	**1.1**
Sp1	2.8 ± 0.2	1.3 ± 0.4	**2.2**

Figure 3. Cell cycle regulation of luciferase constructs containing pentameric binding sites for different transcription factors linked to the CDE–CHR and the *cdc25C* core promoter (–20 to + 121), as indicated at the top of the figure. The experiment was performed with reporter constructs containing either a wild-type or a mutated CDE to be able to determine the contribution of the CDE–CHR module to cell cycle regulation, expressed as the ratio of the values obtained with wild-type to mutant CDE reporter constructs (right-most column). Data are represented as the ratio of luciferase activity in growing versus G_0 cells. Values are given as averages ± SD calculated from two sets of data.

The *cdc25C* CDE–CHR represses specific transactivation domains

In order to analyze the activator specificity of CDE–CHR-mediated repression in more detail we investigated the repressibility of specific transactivation domains fused to a fragment of Gal4 harboring the DNA binding domain (22,23). Plasmid vectors expressing fusion proteins of Gal4 and various activation domains (see Table 1) were co-transfected with a luciferase reporter containing five copies of a Gal4 binding site linked to the *cdc25C* minimal promoter C20 (–20 to +121). Luciferase activities were determined in both growing and quiescent NIH 3T3 cells with both wild-type and mutant CDE reporter plasmids (Fig. 4). CDE–CHR-mediated repression was seen with three different activation domains, the glutamine-rich domains of Oct-2 (Oct-N) (22), Sp1 (Sp1-Q1) (7) and different fragments of the Glu/Ser/Thr-rich activation domain of NF-Y (GAYA-5, -6 and -11) (8,29). The strongest cell cycle regulation was generally seen with GAYA-11, which contains the complete transactivation domain of the A subunit of human NF-Y (corresponding to the B subunit of rat CBF) (9,30).



Type	Activation domain	CDE/CHR-mediated cell cycle regulation	
Gln-rich	Oct-N	2.6 ± 0.4	+
	Sp1-Q1	2.1 ± 0.3	±
	NF-Y (GAYA-6)	2.8 ± 0.2	+
Ser-, Thr-, Gln-rich	NF-Y (GAYA-5)	3.5 ± 0.5	+
	NF-Y (GAYA-11)	5.5 ± 1.5	++
Ser-, Thr-rich	ITF2	1.4 ± 0.3	-
Acidic	VP-16	1.1 ± 0.3	-
	Myc	1.5 ± 0.3	-
Pro-rich	CTF	1.3 ± 0.2	-
-	none (vector control)	1.5 ± 0.3	-

Figure 4. Cell cycle regulation of a luciferase construct containing five copies of a Gal4 binding site linked to the CDE–CHR and the *cdc25C* core promoter (–20 to + 121), as indicated at the top of the figure, after co-transfection of various transactivation domains fused to a Gal4 fragment harboring the DNA binding domain (22,23). The experiment was performed in both growing and G_0 cells with reporter constructs containing either a wild-type or a mutated CDE to be able to determine the contribution of the CDE–CHR module to cell cycle regulation (indicated as ++, +, ± or –). Data are represented as the ratio of the values obtained with wild-type to mutant CDE reporter constructs (as in the right-most column in Fig. 3). Values are given as averages ± SD calculated from five to nine independent experiments. The vector control contained only the Gal4 domain without any fused heterologous sequences.

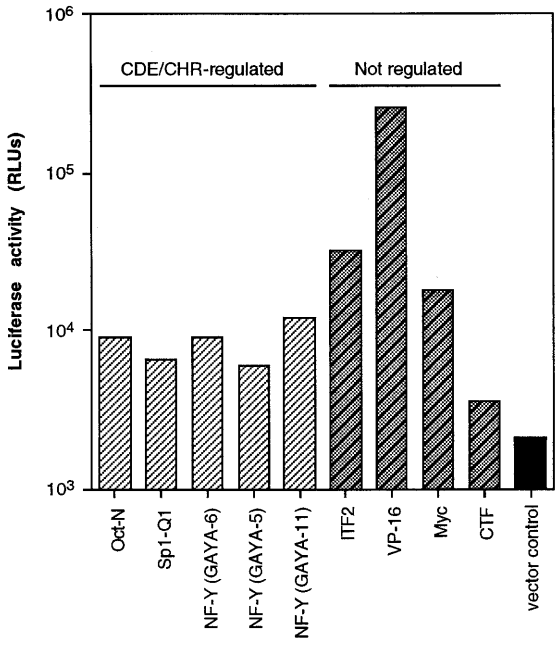


Figure 5. Activation potential of transactivation domains fused to a Gal4 fragment harboring the DNA binding domain after co-transfection with a luciferase construct containing five copies of a Gal4 binding site linked to the CDE–CHR and the *cdc25C* core promoter (–20 to + 121, as in Fig. 4) in growing cells. RLU values are given as averages ± SD calculated from the same experiments as in Figure 4. The vector control contained only the Gal4 domain without any fused heterologous sequencues.

In contrast, no significant cell cycle-regulated repression was detected with other transactivation domains, i.e. ITF2 (31), VP-16 (22,32), Myc (33) and CTF (22,34). These domains are not glutamine rich and belong to the classes of Ser/Thr-rich, acidic or Pro-rich transcription factors (12). These data are in agreement with the results obtained in Figure 3 and confirm the conclusion that CDE–CHR-mediated repression is specific for a subset of transactivation domains. Significantly, the best repression was seen with NF-Y, which is the major transactivator of the *cdc25C* gene and also plays important roles in many other cell cycle genes. The fact that Sp1 (Fig. 3) as well as the Q1 domain of Sp1 (Fig. 4) are less efficiently repressed than NF-Y may, however, not reflect the physiological situation and could rather be due to the artificial experimental set-up, but this question is of minor importance with respect to the conclusions drawn from this study. The same applies to the fact that even the NF-Y-based constructs gave rise to a considerably lower cell cycle regulation than the natural *cdc25C* UAS. In addition, both Sp1 (7) and NF-Y (9) contain multiple activation domains, some of undefined nature, which may all contribute to cell cycle regulation in the context of the natural *cdc25C* promoter.

In general, no correlation was seen between the level of activated transcription and the degree of cell cycle regulation (Fig. 5). Thus, for example, the activation domain showing the best regulation, GAYA-11, gave rise to expression values that were either lower, similar or higher than those seen with the unregulated domains from VP-16, Myc or CTF. Our findings therefore suggest that repressibility (or the lack of it) is an intrinsic property of the activation domains.

Repression by the *cdc25C* CDE–CHR module is orientation dependent

The specificity of repression reported above suggests that the CDE–CHR interacting factor establishes specific contacts with the transcription machinery. If so, the orientation of the CDE–CHR module should be important for its function. We therefore analyzed a series of constructs where the CDE–CHR module was inverted (Fig. 6). This inversion led to a complete abrogation of cell cycle-regulated repression (without affecting transcription levels in growing cells; not shown). The CDE–CHR inversion thus had a similar effect as mutation of the CDE, its inversion (and thus disruption of the repressor module) or mutation of both the CDE and CHR (Fig. 6). We have not formally shown that the inverted CDE–CHR still binds CDF-1, but previously published data strongly suggest that the nucleotides flanking the inverted sequence do not play a role in CDF-1 function (4). The finding that inversion of the CDE–CHR abrogates cell cycle regulation is therefore in line with the conclusion that CDE–CHR-mediated

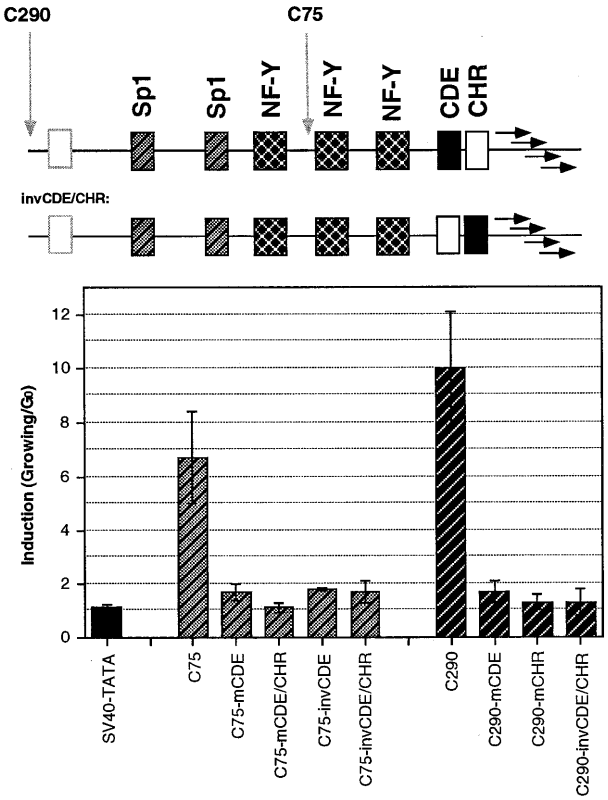


Figure 6. Repression through the CDE–CHR is dependent on its orientation. C290- and C75-based *cdc25C* promoter constructs were assayed in both growing and G_0 cells and the ratio of both values was determined ('induction'). invCDE/CHR, constructs containing inverted CDE–CHR modules as indicated at the top of the figure; invCDE, inversion of the CDE only; mCDE and mCDE/CHR, mutations of the CDE or both the CDE and CHR (controls). The SV40 TATA construct (see legend to Fig. 1) was included for comparison. Values are given as averages ± SD calculated from three independent experiments.

repression appears to involve stereospecific interactions with other transcription factors.

DISCUSSION

The data obtained in the present study suggest that CDE–CHR-mediated repression involves specific protein–protein interactions: (i) the *cdc25C* UAS could not be replaced by heterologous UASs or enhancers without partially or even completely impairing cell cycle-regulated repression; (ii) the glutamine-rich transactivation domains of NF-Y, Sp1 and Oct-2 were repressed in a cell cycle-dependent manner through the CDE–CHR module while several other activation domains did not show a comparable effect; (iii) inversion of the CDE–CHR sequence in the *cdc25C* promoter totally abrogated repression. It is therefore likely that the CDE–CHR binding factor CDF-1 or a protein associated with CDF-1 establishes contacts to a subset of activation domains, their associated co-activators (if any) or specific components of the basal machinery, such as basal transcription factors, TAFs or other cofactors that have been shown to be targeted by glutamine-rich transactivators. In this respect the cofactor PC-4 (35,36), $hTAF_{II}55$ (37) and $hTAF_{II}130$ (38), the human homolog of $dTAF_{II}110$ (39) might be of particular interest. It has been shown that repression by the *Drosophila* protein Krüppel, which depends on interaction with TFIIE, is core promoter dependent and only functions in the context of a TATA box (40). The observation that repression by CDF-1 or associated factors is core promoter independent, at least for the two heterologous core promoters tested, does not rule out the possibility of a direct interaction with the basal machinery, since many of the components contained in the basal complexes formed on different core promoters are identical.

Several distinct mechanisms of transcriptional repression have been proposed (11), including a direct inhibition of general transcription factors (41–43), a local change of chromatin structure near the promoter (44–46) and inhibition of DNA binding by competition or steric hindrance (47,48). The mechanisms underlying transcriptional repression during the cell cycle are poorly understood. Repression of E2F-regulated genes depends on the recruitment of pRb, p107 and p130 to a promoter (49,50). These pocket proteins are able to repress transcription, at least in part, through a position-independent mechanism which apparently involves establishment of interactions with specific transcriptional activators and basal factors (49,51). It is possible that CDF-1 employs a similar mechanism of repression, even though it does not seem to be associated with pocket proteins of the pRb family (see accompanying paper by Liu *et al.*). In agreement with the latter observation, experiments performed with knockout mice that carry disrupted pRb, p107 and/or p130 genes show no change in expression of *cdc25C* (52).

The identification of CDF-1 together with the observations made in this study provide an important basis to investigate these questions in the future. Once CDF-1 is available in purified and/or recombinant form its interactions with the activation domains identified in the present study and with their interaction partners of the basal transcription machinery can be analyzed in detail in order to elucidate the molecular mechanisms underlying CDF-1-mediated repression of transcription during the early phases of the cell cycle.

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