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Cell cycle regulation of the *cyclin A*, *cdc25C* and *cdc2* genes is based on a common mechanism of transcriptional repression

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The S/G_2-specific transcription of the human *cdc25C* gene is due to the periodic occupation of a repressor element ('cell cycle-dependent element'; CDE) located in the region of the basal promoter. Protein binding to the major groove of the CDE in G_0 and G_1 results in a phase-specific repression of activated transcription. We now show that CDE-mediated repression is also the major principle underlying the periodic transcription of the human *cyclin A* and *cdc2* genes. A single point mutation within the CDE results in a 10- to 20-fold deregulation in G_0 and an almost complete loss of cell cycle regulation of all three genes. In addition, the *cdc25C*, *cyclin A* and *cdc2* genes share an identical 5 bp region ('cell cycle genes homology region'; CHR) starting at an identical position, six nucleotides 3′ to the CDE. Strikingly, mutation of the CHR region in each of the three promoters produces the same phenotype as the mutation of the CDE, i.e. a dramatic deregulation in G_0. In agreement with these results, *in vivo* DMS footprinting showed the periodic occupation of the *cyclin A* CDE in the major groove, and of the CHR in the minor groove. Finally, all three genes bear conspicuous similarities in their upstream activating sequences (UAS). This applies in particular to the presence of NF-Y and Sp1 binding sites which, in the *cdc25C* gene, have been shown to be the targets of repression through the CDE. Our results strongly suggest that the CDE/CHR-mediated repression of activation by a specific set of transcription factors is a common mechanism of cell cycle-regulated transcription of S/G_2-specific genes.

Keywords: ATF/*cdc2*/*cdc25C*/*cyclin A*/transcriptional repression

Introduction

In mammalian cells, a specific set of cell cycle genes transcribed around the G_1/S border is regulated by factors of the E2F/DP family (for reviews, see La Thangue, 1994 and Müller, 1995). The E2F/DP transcription factors frequently act as repressors in G_0/early G_1 owing to their association with pocket proteins of the pRb family. In late G_1, the pocket proteins become hyperphosphorylated and dissociate from the complex with E2F/DP, leading to the derepression of E2F-regulated genes. Several genes expressed in late G_1/early S, including B-*myb* (Lam and Watson, 1993), DHFR (Means *et al.*, 1992) and *E2F-1* (Hsiao *et al.*, 1994; Johnson *et al.*, 1994), have been shown to be repressed through an E2F-mediated mechanism in G_0/G_1 and to be derepressed in late G_1. Transcriptional repression is, however, not the only mechanism through which E2F regulates genes. Thus, some E2F-repressed genes, such as B-*myb*, are activated by E2F after the disruption of higher order E2F complexes by viral oncoproteins (Lam *et al.*, 1994). Although there is some evidence for the existence of other cell cycle-controlled transcription factors in mammalian cells, their role in the regulation of specific genes remains to be proven (for a review, see Müller, 1995). However, a novel mechanism of transcriptional repression has recently been described for the human *cdc25C* gene, which shows a clear cell cycle-regulated elevation in expression in late S, with peak levels in G_2 (Sadhu *et al.*, 1990; Lucibello *et al.*, 1995).

We have previously shown that 74 bp of the *cdc25C* upstream sequence are sufficient to confer cell cycle regulation, i.e. induction around late S/G_2, on a luciferase reporter gene (Lucibello *et al.*, 1995). Genomic DMS footprinting revealed the presence of a protein binding site that is located in the region of the basal promoter and is occupied specifically in G_0/G_1. This element, termed 'cell cycle-dependent element' (CDE), contains four G residues that are contacted in the major groove. This pattern of protein binding suggested that the CDE mediates cell cycle regulation through the interaction with a repressor in G_0/G_1. This hypothesis was confirmed by functional assays which showed that mutation of the CDE led to a dramatic deregulation in G_0/G_1. In addition, it was shown that a region of ~60 bp upstream of the CDE was indispensable for both efficient transcription and cell cycle regulation, and both were improved by the presence of another ~100 bp of upstream sequence. This region of the *cdc25C* gene harbours multiple *in vivo* protein binding sites that interact with the constitutive transcriptional activators Sp1 and NF-Y, whose activation function is repressed through a CDE-directed mechanism (J.Zwicker, C.Gross, F.C.Lucibello, M.Truss, F.Ehlert, K.Engeland and R.Müller, submitted). That the CDE-mediated repression is dependent on a functional upstream activating sequence (UAS) could be confirmed by an enhancer swapping experiment: while a fusion construct containing the SV40 early enhancer linked to a *cdc25C* minimal promoter showed a strong cell cycle regulation, no significant regulation was seen with an analogous construct harbouring a mutated CDE (Lucibello *et al.*, 1995).

In this study, we have asked the question whether CDE-

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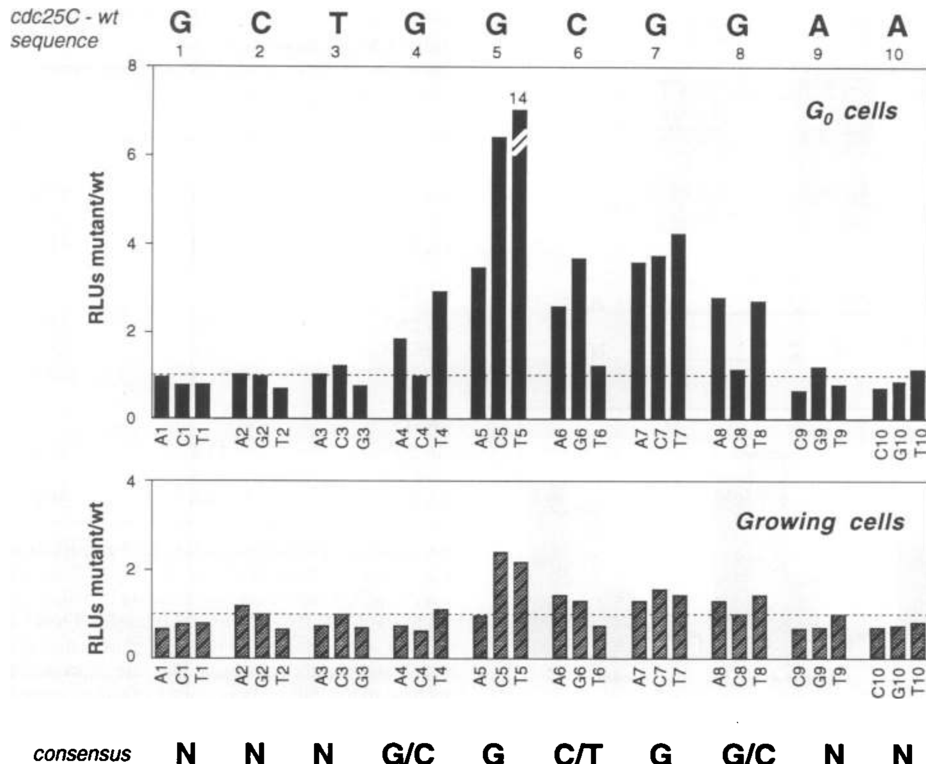

Fig. 1. Structure–function analysis of the CDE. *cdc25C* promoter constructs (based on C75) with single point mutations in the CDE region were analysed in both growing and G_0 cells. Positions 4–8 represent the protected CDE core. Nucleotide 1 represents position −19 in the *cdc25C* promoter. Results of transient luciferase assays are expressed as the activity of the respective mutant relative to the wild-type C75 construct. All values are standardized relative to the activity of a SV40 reporter. The results shown in the figure corroborate the data of 14 independent experiments. Each construct was assayed 4–8 times using at least two independent preparations of plasmid DNA. Values represent averages. The SD was in the range of 5–30%. The consensus sequence shown at the bottom incorporates all nucleotides that lead to a <1.5-fold deregulation.

mediated repression might play a similarly crucial role in the periodic transcription of other cell cycle genes. The relevance of this question was emphasized by comparing the sequence around the CDE with the promoter sequences of other S/G_2-specific genes. This comparison yielded striking identities within two distinct regions of three different genes: *cdc25C*, *cyclin A* and *cdc2*. One of these regions is the CDE itself, and a second novel sequence was found at an identical short distance downstream of the CDE. This element was termed 'cell cycle genes homology region' (CHR). Significantly, structure–function analyses showed that the differences in (and around) the *cdc2*, *cyclin A* and *cdc25C* CDEs have no effect on their function as cell cycle-regulated repressor elements, while multiple base changes in either the CDE or the CHR lead to deregulation in G_0/G_1. In addition, genomic footprinting of the *cyclin A* promoter showed cell cycle-regulated protein binding to the major groove of the CDE and the minor groove of the CHR. We also note that all three genes show a conspicuous preference for Sp1 and NF-Y binding sites in their UAS, elements that have been shown to be required for repression of the *cdc25C* gene. It is therefore likely that the molecular basis for CDE-mediated negative regulation is a conserved mechanism involving the repression of certain transcriptional activators like Sp1 and the CCAAT-box binding factor NF-Y. Based on these observations, we propose that CDE–CHR-based repression is a common regulatory mechanism for S/G_2-specific genes.

Results

Mutagenesis of the cdc25C CDE: delineation of a consensus sequence

In order to delineate the borders of the CDE, and to identify critical nucleotide positions and permissive base changes, we performed saturation mutagenesis of the protected residues in the CDE and the flanking sequences (Lucibello *et al.*, 1995). The entire sequence from −19 to −10 was mutated, introducing every possible base exchange in the *cdc25C* promoter–luciferase construct C75, which extends to position −75 at the 5′ end. To facilitate reference to specific positions within the CDE, especially in the context of other promoters, we defined these nucleotides as CDE positions 1–10 (see Figure 1). All 30 mutant constructs were transiently transfected into NIH3T3 cells, and luciferase activity was determined in both G_0 and growing cells relative to the wild-type promoter–luciferase construct. The data in Figure 1 show that no significant deregulation was seen when point mutations were introduced at positions 1, 2, 3, 9 or 10. In contrast, strong effects were seen when bases at positions 4–8 were altered. These core positions correspond exactly to the residues found to be protected in genomic footprinting experiments. Positions 5 and 7 were found to be the most critical ones, since any alteration here led to a clear deregulation, i.e. loss of repression in G_0. A G→C transversion at position 4 and a C→T transition at position 6 were permissive, while any other

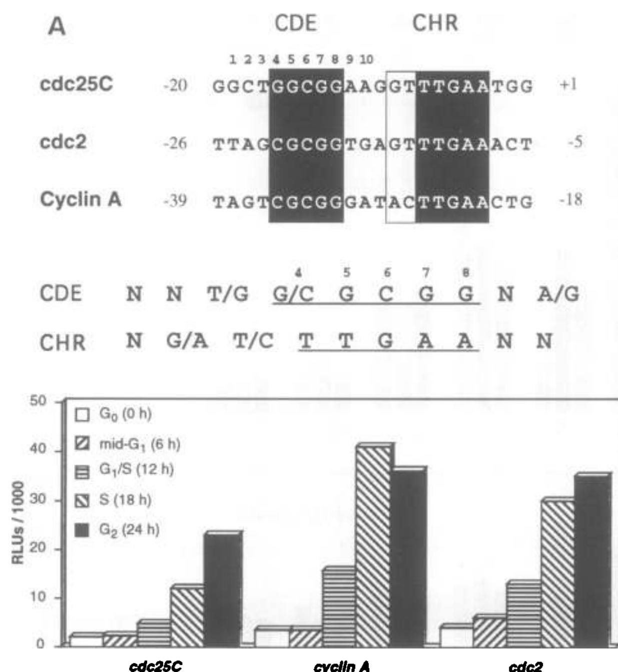

Fig. 2. (**A**) Alignment of *cdc25C*, *cyclin A* and *cdc2* promoter sequences in the regions of the CDE and CHR elements (highlighted by black boxes). The numbers at the top of the *cdc25C* and CDE sequences follow the same system as in Figure 1. Core sequences are underlined (based on the functional data in Figure 1 for the CDE or on identities for the CHR). (**B**) Induction kinetics of *cdc25C* (C290), *cyclin A* and *cdc2* promoter–luciferase constructs after serum stimulation of quiescent NIH3T3 cells. Measured activities (RLUs) were standardized using a SV40 early promoter construct. Progression through the cell cycle was measured by FACS analysis and incorporation of 5-bromodeoxyuridine. After 12, 18 and 24 h, the majority of the cells were found to be at the G_1/S transition, in S-phase and in G_2, respectively.

alteration led to a clear deregulation. As expected, deregulaion was particularly strong in G_0 cells (2- to 14-fold), while the effects were much less dramatic in growing cells (≤2.5-fold). These results are in agreement with previous observations which showed that the CDE acts as a repressor element specifically in G_0 and G_1 cells. From these results, the consensus sequence N N N G/C G C/T G G/C N N can be derived. This sequence incorporates all nucleotides that led to a ≤1.5-fold deregulation in G_0 cells.

Identification of two cooperating repressor elements, CDE and CHR, in the cdc25C, cyclin A and cdc2 promoters

We next performed an alignment of the *cdc25C* promoter sequence in the region of the CDE with other cell cycle genes. As shown in Figure 2A, significant similarities were seen with both the *cdc2* (Dalton, 1992; Furukawa *et al.*, 1994) and *cyclin A* (Henglein *et al.*, 1994) genes. Both genes show a deviation at position 4 in the CDE from a G to a C which, according to the results in Figure 1, is perfectly permissible. In addition to the striking similarity in the region of the CDE, we identified a second element downstream of the CDE which is conserved between all three genes. Thus, a 7 bp stretch (GTTTGAA) starting four nucleotides 3′ to the CDE is identical among *cdc25C* and *cdc2*. Moreover, the last five nucleotides of this region are identical in *cdc25C*, *cyclin A* and *cdc2*. We termed this region CHR. The fact that the spacing between the CDE and the CHR is identical in all three genes, and that these elements occur at similar positions within the basal *cdc25C*, *cyclin A* and *cdc2* promoters, clearly suggests that the CDE/CHR region fulfils a similar function in all three genes.

Table I. Deregulation of the *cdc25C*, *cyclin A* and *cdc2* promoters by mutations in the CDE or CHR repressor elements

	G_0	Growing	Factor
wt			
cdc25C	0.8	13.1	17.5
cyclin A	0.7	27.1	41.7
cdc2	1.0	41.2	41.2
mCDE			
cdc25C	7.6	11.6	1.5
cyclin A	13.4	23.9	1.8
cdc2	11.3	33.9	3.0
mCHR			
cdc25C	14.4	21.0	1.5
cyclin A	15.5	28.3	1.8
cdc2	18.6	38.6	2.1

wt: wild-type promoters; mCDE: C75 constructs with mutated CDEs (G→T transversions at –13 in *cdc25C*, –33 in *cyclin A* and –19 in *cdc2*); mCHR: constructs with mutated CHRs (mutation of positions –6 to –3 in *cdc25C*, –26 to –23 in *cyclin A* and –12 to –9 in *cdc2*). Results were obtained by transient transfection of NIH3T3 cells with luciferase reporter constructs. Data are expressed as RLUs/1000 relative to a SV40 early promoter/enhancer reporter construct used for standardization. The values given in the table represent the average of two independent experiments. The SD of normalized values (relative to *cdc25C*-wt) was ≤20%.

To address this point experimentally, we first cloned the *cdc2* and *cyclin A* promoters by a polymerase chain reaction (PCR)-based strategy and placed them in front of a luciferase reporter gene to be able to analyse their regulation during the cell cycle in comparison with *cdc25C*. The data in Figure 2B show that the *cyclin A* and *cdc2* promoters were coordinately activated during the cell cycle, preceding activation of *cdc25C*. Thus, at 12 h post-stimulation of quiescent cells, i.e. around the G_1/S transition, *cdc25C*–luciferase activity was elevated ~2-fold, while both other promoters showed an induction of ~4-fold. In addition, *cdc25C* transcription increased from 18 to 24 h, i.e. in G_2, while the activity of both *cyclin A* and *cdc2* reached a plateau in S-phase. These observations are in agreement with the cell cycle regulation observed with the endogenous *cdc25C*, *cyclin A* and *cdc2* genes, showing that the promoter fragments used in this study largely reflect the physiological situation.

In order to assess the functional relevance of the CDE and CHR elements in the cell cycle-regulated transcription of the *cyclin A*, *cdc2* and *cdc25* genes we generated a series of mutant constructs with all three promoters. In addition to the respective wild-type constructs, mutants in the CDE (point mutation at position 7) and in the CHR (alteration of the conserved TGAA sequence) were tested in transient luciferase assays in both G_0 and growing NIH3T3 cells (Table I). As expected, the wild-type constructs showed very little activity in G_0, but a clear induction in growing cells (17.5- to 41.7-fold). Strikingly,

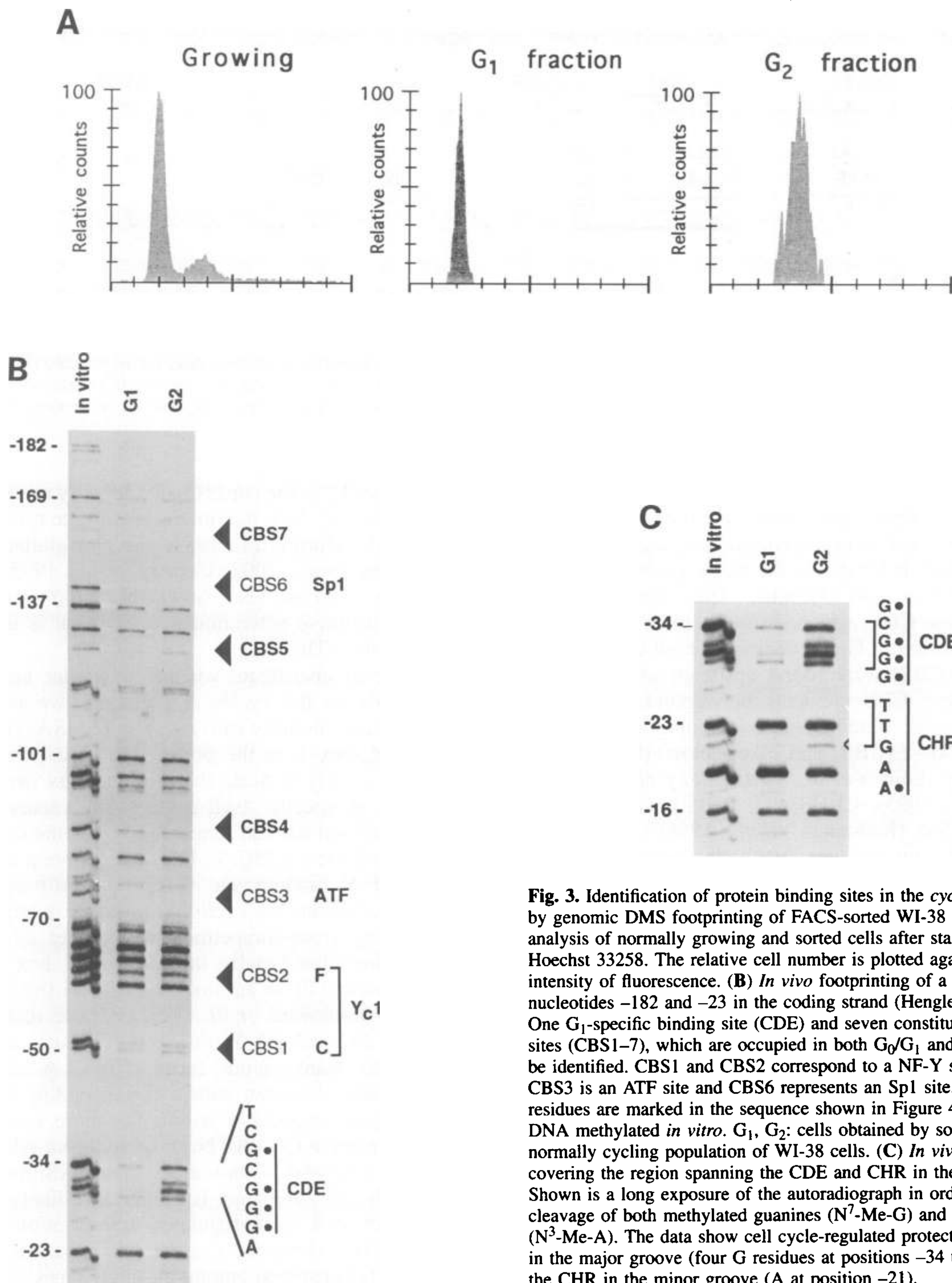

Fig. 3. Identification of protein binding sites in the *cyclin A* promoter by genomic DMS footprinting of FACS-sorted WI-38 cells. (**A**) FACS analysis of normally growing and sorted cells after staining with Hoechst 33258. The relative cell number is plotted against the intensity of fluorescence. (**B**) *In vivo* footprinting of a region spanning nucleotides –182 and –23 in the coding strand (Henglein *et al.*, 1994). One G_1-specific binding site (CDE) and seven constitutive binding sites (CBS1–7), which are occupied in both G_0/G_1 and G_2 cells, can be identified. CBS1 and CBS2 correspond to a NF-Y site (Y_c-box 1), CBS3 is an ATF site and CBS6 represents an Sp1 site. All protected G residues are marked in the sequence shown in Figure 4. *In vitro*: naked DNA methylated *in vitro*. G_1, G_2: cells obtained by sorting of a normally cycling population of WI-38 cells. (**C**) *In vivo* footprint covering the region spanning the CDE and CHR in the coding strand. Shown is a long exposure of the autoradiograph in order to detect cleavage of both methylated guanines (N^7-Me-G) and adenines (N^3-Me-A). The data show cell cycle-regulated protection of the CDE in the major groove (four G residues at positions –34 to –30) and of the CHR in the minor groove (A at position –21).

any of the mutations in the CDE or CHR led to an almost complete loss of repression in G_0 cells (10- to 22-fold), while affecting the activity in growing cells only to a marginal extent (<2-fold). As a consequence, with each promoter cell cycle regulation was impaired upon mutation of the CDE or the CHR (see the right-most column of Table I). These results clearly establish that the CDE and CHR are the major regulatory elements conferring cell cycle regulation on the *cdc25C*, *cyclin A* and *cdc2* promoters. In addition, these observations indicate that the CDE and CHR cooperate in repressing transcription, since neither of the two elements works to a significant extent on its own.

Cell cycle-regulated protection of the CDE in the major groove and of the CHR in the minor groove in vivo

The CDE in the *cdc25C* promoter is bound by protein specifically in G_0/G_1, as shown by genomic DMS footprinting (Lucibello *et al.*, 1995). In order to obtain further evidence that CDE-mediated repression is a common mechanism of cell cycle-regulated transcription, we performed *in vivo* footprinting (Pfeifer *et al.*, 1989) on the *cyclin A* promoter. For this purpose, normally cycling WI-38 human fibroblasts were treated with DMS and sorted by FACS. As shown in Figure 3A, highly pure preparations of G_1 and G_2 cells could be obtained by this procedure

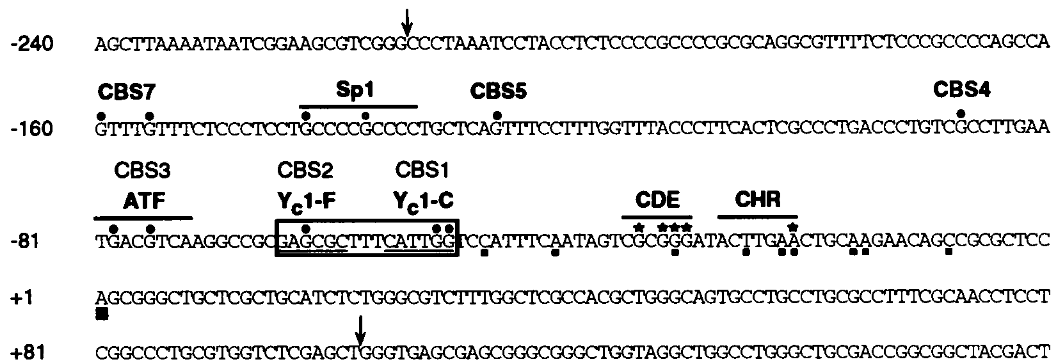

Fig. 4. *In vivo* protein binding sites in the *cyclin A* upstream region (Henglein *et al.*, 1994). Protected G residues detected by genomic DMS footprinting (see Figure 3) are marked by filled circles and asterisks to denote constitutive and cell cycle-regulated protection, respectively. The sites of transcription initiation are marked by solid squares, the major initiation site being indicated by a large square. Y_c-boxes 1 are shaded. Arrows show the 5′ end points of the promoter construct used in this study.

(<10% contamination). Footprinting analysis of ~180 nucleotides of upstream sequences led to the identification of eight regions that showed protection of the N-7 atom of guanine, which is located in the major groove (Figures 3B and 4) (Thanos and Maniatis, 1992). One of these elements was the CDE, which showed a clear cell cycle-regulated protection in G_1. In addition, seven constitutive binding sites (CBS) were found upstream of the CDE. Several of these CBS elements correspond to known transcription factor binding sites, including recognition sequences for NF-Y (CBS1 and 2; see below) (Dorn *et al.*, 1987; van Hujisduijnen *et al.*, 1990; Maity *et al.*, 1992; Coustry *et al.*, 1995), CREB/ATF (CBS3) (Ziff, 1990) and Sp1 (CBS6) (Kadonaga *et al.*, 1986). On longer exposures of the autoradiographs, the less efficient cleavage of methylated adenine (*N*-3-methyladenine) also became clearly detectable, and led to the identification of an A residue at position –21 showing cell cycle-regulated protection (Figures 3C and 4). This contact occurs, as in the case of the major groove contacts in the CDE, specifically in G_1, and indicates a minor groove contact within the CHR, since the N-3 atom of adenine is located in the minor groove (Thanos and Maniatis, 1992). The *in vivo* footprinting result is thus in perfect agreement with the functional analysis shown in Table I. Both the CDE and the CHR show protein binding *in vivo* specifically in G_1 (Figures 3B and 4) and in G_0 (data not shown), and both elements are required for cell cycle-regulated repression. No other footprints indicating major or minor groove contacts were detected in the *cyclin A* promoter between positions –186 and +26 (Figures 3 and 4, and data not shown).

Identification of NF-Y as a cyclin A UAS binding factor: similarities in the activation mechanisms among different CDE/CHR-regulated genes

As shown in Figure 5A, CBS1 encompasses a reverse CCAAT box and shows a strong similarity with the CBS1 element in the *cdc25C* gene (Lucibello *et al.*, 1995). In addition, the adjacent element, CBS2, also shares sequence homology among the two genes and both these elements share three protected G residues. Moreover, in both genes, CBS1 and 2 occur at a similar distance upstream of the CDE, suggesting that they may serve similar functions. We have previously shown (J.Zwicker *et al.*, submitted) that CBS1 and 2 in the *cdc25C* gene actually represent one binding site, a Y_c-box, that interacts with the transcription factor NF-Y (Dorn *et al.*, 1987; van Hujisduijnen *et al.*, 1990; Maity *et al.*, 1992; Coustry *et al.*, 1995). In the same study, we have also shown that NF-Y is endowed with a constitutive activation potential that is under the control of the CDE.

In order to investigate whether a similar mechanism might apply to the *cyclin A* promoter, we performed electrophoretic mobility shift analyses (EMSAs) using the *cyclin A* Y_c-box 1 as the probe. The data presented in Figure 5B clearly indicate that the complex detected by this probe is specific (self-competition versus random competition) and has the same mobility as the complexes forming with the *cdc25C* Y_c-box 1 and a bona fide high-affinity NF-Y binding site (Eα/Y) (Mantovani *et al.*, 1992). In addition, the *cyclin A* Y_c-box 1 complex was abolished by cross-competition with either of the two other binding sites. Finally, the *cyclin A* Y_c-box complex was supershifted by an antibody specific for the B subunit of NF-Y (Mantovani *et al.*, 1992). These data clearly show that the *cyclin A* Y_c-box 1 (CBS1/2) is a binding site for the transcription factor NF-Y. A significant similarity was also seen with a corresponding region of the *cdc2* gene, especially within the more crucial core sequence (reverse CCAAT box). In addition, *cdc2* seems to contain a second Y_c-box at a similar position as that found in the *cdc25C* gene. It is therefore likely that the principle of cell cycle-regulated transcription through the CDE/CHR elements, i.e. the repression of specific activators, is conserved among all three genes.

Discussion

The human *cdc25C* gene is expressed in a phase-specific manner with a clear maximum level of transcription in G_2 (Sadhu *et al.*, 1990; Lucibello *et al.*, 1995). The molecular basis for this periodic transcription is the phase-specific association and dissociation of a repressor protein, or protein complex, with a regulatory element (CDE) located in the region of the basal promoter (Lucibello *et al.*, 1995). This mechanism is clearly different from the 'classical' E2F-mediated regulation, since E2F complexes bind DNA throughout the cell cycle (Plet *et al.*, 1992; La Thangue, 1994; Müller, 1995). In addition, in contrast to

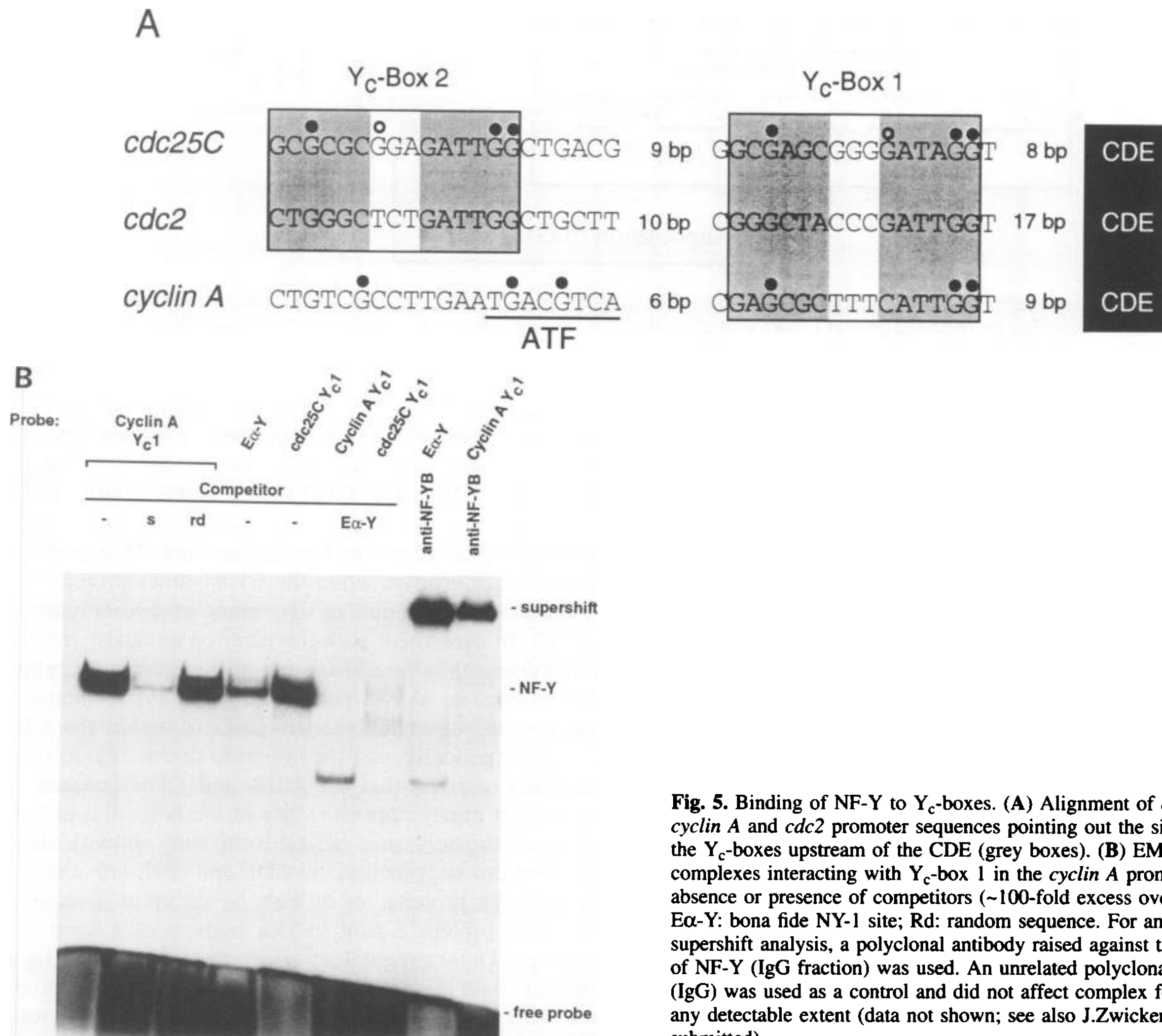

Fig. 5. Binding of NF-Y to Y_c-boxes. (**A**) Alignment of *cdc25C*, *cyclin A* and *cdc2* promoter sequences pointing out the similarities in the Y_c-boxes upstream of the CDE (grey boxes). (**B**) EMSA of protein complexes interacting with Y_c-box 1 in the *cyclin A* promoter in the absence or presence of competitors (~100-fold excess over probe). Eα-Y: bona fide NY-1 site; Rd: random sequence. For antibody supershift analysis, a polyclonal antibody raised against the B subunit of NF-Y (IgG fraction) was used. An unrelated polyclonal antibody (IgG) was used as a control and did not affect complex formation to any detectable extent (data not shown; see also J.Zwicker *et al.*, submitted).

a bona fide E2F site, the CDE shows only a weak interaction with recombinant GST-E2F-1 protein (kindly provided by L.Bandara and N.La Thangue; K.Engeland, unpublished observations).

The main goal of the present study was to investigate whether CDE-mediated repression might also be involved in the regulation of other cell cycle genes. Towards this end, we performed a comparison of the sequence around the CDE with the basal promoter regions of other S/G_2-specific genes, which revealed conspicuous identities within two distinct regions of the *cdc25C*, *cyclin A* and *cdc2* genes. One of these regions is the CDE itself, and a second novel sequence, termed CHR, was found at an identical distance downstream of the CDE (Figure 2A). Several lines of evidence demonstrate that both these elements play a crucial role in cell cycle-regulated repression. (i) Structure–function analyses showed that the differences in (and around) the *cdc25C*, *cyclin A* and *cdc2* CDEs have no effect on their function as cell cycle-regulated repressor elements (Figures 1 and 2). (ii) On the other hand, different point mutations within the conserved core of the CDE led to a loss of repression in G_0 (Figure 1 and Table I). (iii) Genomic footprinting of the *cyclin A* promoter showed cell cycle-regulated protein binding to the same nucleotides in the major groove (Figure 3B). (iv) Alteration of the CHR sequence by a 4 bp substitution led to a similar deregulation in G_0 as mutations in the CDE core in all three promoters (Table I). (v) This cell cycle-associated function of the CHR correlates with the periodic occupation of the CHR, but in contrast to the CDE–protein interaction described above, these contacts occur in the minor groove (Figure 3C). These observations clearly suggest that CDE–CHR-based repression is a common regulatory mechanism for S/G_2-specific genes.

Our results are in agreement with published observations, showing that regions of the *cyclin A* and *cdc2* promoters containing the CDE/CHR regions are able to confer cell cycle regulation on a reporter gene (Dalton, 1992; Furukawa *et al.*, 1994; Henglein *et al.*, 1994). Both studies failed, however, to recognize these sites as the elements necessary for cell cycle regulation. In the case of the *cdc2* promoter, it has been suggested that a potential E2F site located upstream of the CDE is responsible for cell cycle regulation (Dalton, 1992; Furukawa *et al.*, 1994), but this hypothesis is based on deletion analyses and *in vitro* protein binding studies only. The effect of a point mutation in the potential E2F site has, however, been investigated in a very recent study. This work showed that mutation of the E2F site led to a 1.8-fold reduction in the level of cell cycle-induced transcription (Shimizu *et al.*, 1995), demonstrating that the E2F site does not

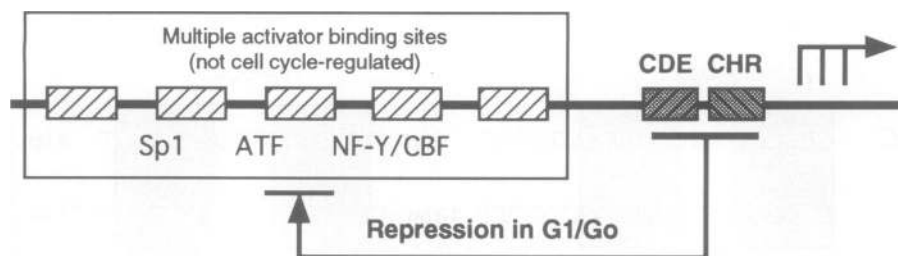

Fig. 6. Model of CDE-mediated repression of activated transcription. The hallmark of this model is the CDE/CHR-directed repression of the activation function of a specific set of transcriptional factors with glutamine-rich activation domains, such as Sp1, NF-Y and CREB/ATF family members.

indeed play a major role in the periodic transcription of *cdc2*. An essential function for the CDE/CHR elements is also suggested by a recent study showing TPA-mediated repression of the *cdc2* gene via a proximal promoter fragment harbouring the CDE and CHR, but not the E2F site (Sugarman *et al.*, 1995). On the other hand, Shimizu *et al.* (1995) showed that mutation of the E2F site (in the presence of an intact CDE–CHR region) led to a delayed induction of *cdc2* transcription during the cell cycle. These delayed kinetics closely resemble those of the wild-type *cdc25C* promoter (Lucibello *et al.*, 1995). This finding would be consistent with the idea that the CDE–CHR in the *cdc2* and *cdc25C* genes acts in a very similar manner, and that the earlier induction of *cdc2* is due to a least two superimposed effects, i.e. (i) derepression and/or activation via the E2F site in late G_1 and (ii) derepression of the CDE in S/G_2. In agreement with this model, *cyclin A*, which is co-expressed with *cdc2*, also harbours potential E2F sites downstream of the CHR (Henglein *et al.*, 1994). The possibility of a potential cooperation of E2F sites and CDE/CHR elements is an interesting hypothesis that will be tested in further detail in future experiments. It will also be of particular interest to investigate whether the CDE–CHR elements, although acting through a similar mechanism in all three genes, interact with related, but distinct proteins.

It is also possible that upstream regulators contribute to cell cycle regulation to some extent. This is suggested by recent observations indicating that the *cyclin A* CREB/ATF site shows a cell cycle-regulated response to stimulation of the cAMP signal transduction pathway (Desdouets *et al.*, 1995) and mediates the downregulation of *cyclin A* in contact-inhibited endothelial cells (Yoshizumi *et al.*, 1995). It has, however, to be noted that any loss of activation also diminishes the extent of cell cycle-regulated repression of upstream activators through the CDE–CHR elements, as shown for the *cdc25C* promoter (J.Zwicker *et al.*, submitted), suggesting that the effects observed via the CREB/ATF site may be in part indirect and dependent on the CDE–CHR region. In agreement with such an indirect effect is the lack of cell cycle regulation in constructs lacking functional CDE and CHR elements in spite of the presence of an intact CREB/ATF site (Table I).

At present, we have not identified the factor(s) interacting with the CDE and CHR, although the weak interacton of GST-E2F-1 with the CDE (see above) might indicate the involvement of an E2F-related factor. Our results indicate that both sites functionally cooperate, since neither the CDE nor the CHR are able to repress transcription to any significant extent on their own. The fact that both elements are functionally non-separable, taken together with their contiguity, suggests that they might interact with a common protein complex. Interestingly, the CDE and CHR differ substantially in the location of protein contacts. As shown by genomic DMS footprinting of the *cyclin A* promoter, the CDE is protected in the major groove, while the CHR shows protection of at least one A residue in the minor groove (Figure 3B and C). In agreement with the latter observation, the only G residue within the CHR is not protected in either *cyclin A* (Figure 3) or *cdc25C* (Lucibello *et al.*, 1995), indicating the absence of major groove contacts within the CHR. Computer modelling of the structure in this region of the promoter showed that the CHR and CDE contacts are located on nearly opposite sides of the helix at a distance of approximately one helical turn (not shown). These observations suggest that the CDE and CHR are contacted by different proteins, or at least by different domains of the same protein. Examples for both models have been reported. Thus, a regulatory site in the human IFN-β gene interacts with NF-κB through contacts in the major groove and with HMGI(Y) through contacts in the minor groove (Thanos and Maniatis, 1992). In contrast, homeodomain proteins have been reported to establish contacts to both the major and minor groove through different domains of the same protein (Otting *et al.*, 1990; Harrison, 1991). These questions can be addressed only once the CDE/CHR-binding protein(s) have been identified.

A comparison of the *cdc25C*, *cyclin A* and *cdc2* promoters also showed a conspicuous preference for Sp1 and NF-Y binding sites in the UAS of all three genes. This is of particular interest, since these elements have been shown to be required for the CDE/CHR-mediated repression of the *cdc25C* gene, which harbours one Sp1 site and three NF-Y binding sites (Y_c-boxes) within the 150 bp upstream of the CDE (Lucibello *et al.*, 1995; J.Zwicker *et al.*, submitted). Importantly, the combination of either the Sp1 site or two Y_c-boxes with the CDE/CHR elements is sufficient for cell cycle-regulated transcription. Likewise, we have identified by genomic footprinting a bona fide Sp1 site at position −143 to −134 in the *cyclin A* promoter (Figures 3B and 4). Another potential Sp1 site at position –66 to −173 was not protected and therefore presumably not relevant. In addition, we have identified both by *in vivo* footprinting (Figures 3B and 4) and in biochemical studies (Figure 5B) a Y_c-box at position −52 to –66, which corresponds almost exactly to the position of the Y_c-box 1 in the *cdc25C* gene (Figure 5A). The *cyclin A* upstream sequence also showed a clear footprint on a potential CREB/ATF binding site at position −74 to −81, as well

as on one G residue immediately upstream (Figures 3B and 4). It is possible that regulation via this site contributes to the precise timing of *cyclin A* induction during the cell cycle, as discussed above for the potential involvement of E2F sites. Two Y_C-box-like regions were also found in the *cdc2* upstream sequence, again at similar locations relative to the CDE as in *cdc25C* (one helical turn further upstream; Figure 5A). It is therefore likely that the molecular basis for CDE/CHR-mediated negative regulation is a conserved mechanism, as outlined in the model shown in Figure 6. The hallmark of this model is the CDE/CHR-directed repression of the activation function of a specific set of transcription factors. Interestingly, the transcription factors interacting with the *cdc25C*, *cyclin A* and *cdc2* UAS, i.e. Sp1, NF-Y and CREB/ATF family members, all share glutamine-rich activation domains (Courey and Tjian, 1988; Gonzalez *et al.*, 1991; Li *et al.*, 1992; Coustry *et al.*, 1995). These similarities point to a common target of CDE/CHR-mediated repression, perhaps a mechanism that links these activators to the basal machinery (Tjian and Maniatis, 1994).

Materials and methods

Cell culture, DNA transfection and luciferase assays

WI-38 cells (Hayflick, 1965) were obtained from the ATCC and NIH3T3 cells were kindly provided by R.Treisman (ICRF, London). NIH3T3 cells were cultured in Dulbecco–Vogt modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. WI-38 cells were maintained in a medium containing DMEM and MCDB105 at a 1:1 ratio. For synchronization in G_0, cells were maintained in serum-free medium for 2 days. NIH3T3 cells were transfected by the DEAE–dextran technique (Lucibello *et al.*, 1995). Determination of luciferase activity was performed as published elsewhere (Herber *et al.*, 1994; Lucibello *et al.*, 1995). SV40 reporter constructs were used to standardize the results, and to correct for differences among growing and quiescent cells. Final results were expressed as RLUs/2×10^5 transfected recipient cells.

Sequence analysis and luciferase constructs

DNA sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using Sequenase (USB). Ambiguous sequences and GC-rich stretches were verified by 'cycle sequencing' using *Tth* polymerase (Pharmacia). *cyclin A* (−214 to +100) (Henglein *et al.*, 1994) and *cdc2* (−249 to +92) (Furukawa *et al.*, 1994) promoter fragments were generated by PCR with compatible ends for cloning into the promoterless luciferase vector pXP2 (Nordeen, 1988). All PCR-amplified fragments were verified by DNA sequencing.

Genomic footprinting

Genomic footprinting (Pfeifer *et al.*, 1989) of the *cyclin A* gene in WI-38 cells and cell sorting by FACS were performed as described (Lucibello *et al.*, 1995). The following oligonucleotides were used as primers:

Primer 1,
TM = 54°C, 5′-d(AGCCCAGGCCAGCCTA)-3′;
Primer 2,
TM = 74°C, 5′-d(CAGCCCGCCCGCTCGCTCACC)-3′;
Primer 3,
TM = 84°C, 5′-d(GCTCACCCAGCTCGAGACCACGCAG)-3′.

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