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In vivo structure of the cell cycle-regulated
human cdc25C promoter

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SUMMARY

The *cdc25C* promoter is regulated during the cell cycle by the transcriptional repressor CDF-1 which inhibits the activation function of transcriptional activators bound upstream, most notably NF-Y/CBF. Here, we have analyzed in detail the in vivo structure of the *cdc25C* promoter. Micrococcus nuclease and methidiumpropyl-EDTA footprinting strongly suggest that the proximal promoter encompassing the CDE-CHR and the upstream NF-Y sites is organized in a positioned nucleosome throughout the cell cycle. Furthermore, structural perturbations were detected by DNase I, phenanthroline copper (OP-Cu) and KMnO₄ footprinting at the NF-Y binding sites in vivo, which is in agreement with the reported property of NF-Y to bend DNA in vitro. Similar results were obtained with the structurally and functionally related *cyclin A* promoter. The structural perturbations seen in DNase I and OP-Cu footprints were less pronounced in G₀ cells compared to cycling cells, which presumably reflects a weakened in vivo interaction of NF-Y with its cognate DNA element in G₀. It is likely that these structural perturbations together with the reported ability of NF-Y to recruit HAT activity contribute to an opened chromatin structure as a prerequisite for an optimal regulation through activation and repression.
INTRODUCTION

NF-Y/CFB is a ubiquitously expressed transcriptional activator that interacts with CCAAT-boxes found in the promoters of a wide variety of genes, including hormone inducible, developmentally controlled and cell cycle regulated genes (1-4). A particularly well studied example of the latter group of genes is the G2-specific cdc25C promoter (5-10) which contains three functionally important NF-Y binding sites. Genomic DMS footprinting and functional promoter analyses demonstrated that NF-Y binding to the core and flanking regions of these NF-Y sites is necessary for both maximal promoter activity and cell cycle regulation (6). The cdc25C promoter is regulated by the transcriptional repressor CDF-1 that binds to a bipartite DNA element (CDE-CHR) in G1/G2 (9), thereby blocking the function of the NF-Y and Sp1/Sp3 complexes bound immediately upstream of the CDE-CHR (6). Upon entry into S/G2, the interaction of CDF-1 to its cognate binding site is abrogated, thus allowing for the NF-Y and Sp1/Sp3 mediated transcriptional activation of the cdc25C gene (5, 6, 9). A similar situation exists in case of the cyclin A promoter which is also repressed by CDF-1 and activated by NF-Y (7).

The mechanism through which NF-Y activates transcription is not fully understood. However, NF-Y has been reported to interact with the histone acetylases Gcn5 and P/CAF (11) and these interactions seem to be relevant in the context of the multiple drug resistance-1 gene promoter (12). Furthermore, it has been shown that NF-Y is capable of associating with nucleosomal templates in vitro (13), although the in vivo relevance of this observation remains to be investigated. These observations imply a role for NF-Y in chromatin remodeling and could possibly provide an explanation for
the ability of DNA-bound NF-Y to recruit other transcription factors to promoter DNA (14, 15). NF-Y binds to both the major and minor groove of the DNA and has been reported to induce DNA-bending in vitro (16) which is believed to be important for the functional organization of activated promoters in vivo, as in the case of the γ-globin promoter (17). However, there is no evidence that NF-Y binding to promoter DNA is indeed associated with structural distortions in vivo.

In the present study we have used a combination of different genomic footprinting techniques to analyze in detail the in vivo structure of the cdc25C promoter, in particular with respect to its nucleosomal organization and transcription factor-associated structural distortions.

**EXPERIMENTAL PROCEDURES**

*Cell culture and synchronisation* — WI-38 cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle medium and MCDB 135 medium with 10% fetal calf serum. For synchronization in G0, cells were maintained in serum-free medium for 3 days.

*Genomic footprinting* — For DMS footprinting WI-38 cells were grown to 70% confluency. After treatment with 0.2% DMS for 2 min the cells were washed three times with cold PBS and the DNA was isolated using DNAzol (Gibco-BRL). For potassium permanganate (KMnO4) treatment, the cells were incubated with 20 mM KMnO4 for 2 min and were washed twice with PBS containing 2% β-mercaptoethanol and once with
PBS. For DNase I, Mnase, MPE and OP-Cu footprinting, the cells were scraped into PBS and resuspended in DNase I digestion buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 0.1 mM PMSF, 1M sucrose) containing 0.2 % NP-40 (for permeabilization of cells). For MNase and MPE cleavage, the scraped cells were homogenized on ice with 10 strokes in a Dounce homogenizer. MNase treatment was performed with 0.05-1 U of enzyme for 3 min. For DNase I cleavage, 200-400 U of enzyme (Boehringer, Mannheim) were added, the reactions were stopped after 5 min by addition of 1/5 volume of 62.5 mM EDTA and 2.5% SDS. MPE treatment was performed as described (18). For OP-Cu treatment, a complex formed from 40 µM 1,10-phenanthroline and 10 µM CuSO₄ (final concentrations) was added to the cell suspension and the reaction was started by the addition of 3-mercaptopyruvate acid to a concentration of 6.9 mM. After 2-4 min the reaction was stopped by addition of 2.9-dimethyl-1,10 phenanthroline to a final concentration of 2.8 mM. For comparison, WI-38 genomic DNA was either methylated in vitro with 0.2% DMS for 10-30 sec, treated with 2 mM KMnO₄ for 30 sec, cleaved with 0.05-0.1 U MNase for 30 sec or with 2-4 U DNase I for 30 s, or treated with OP-Cu for 60-75 sec. In each case, 3 µg of piperidine-cleaved genomic DNA were used for LM-PCR with the Stoffel fragment of Taq polymerase (Perkin Elmer) as described (5). For OP-Cu and MNase treatment, the DNA was phosphorylated with T4 polynucleotide kinase (New England Biolabs) prior to LM-PCR. Samples were phenol extracted and ethanol precipitated after primer extension with ³²P-labelled primers. The following oligonucleotides were used as primers:

**cdc25C** promoter, primer set TS1:

- primer 1: 5'-d(AGGGAAAGGAGGTAGTT)-3';
- primer 2: 5'-d(TAGATTGCAGCTATGCCTTCGAC)-3';
primer 3: 5’-d(CCTTCCGACTGGGTAGGCAACGTCG)-3’.

cdc25C promoter, primer set TS2:

primer 1: 5’-d(CTGCCTAGCAATTCTCC)-3’,

primer 2 5’-d(TGGCTATCGTTGGCTGAGCAG)-3’,

primer 3: 5’-d(GGGCTCGAGATCACCTGGGGGCGC)-3’.

cdc25C promoter, primer set BS:

primer 1: 5’-d(CACTAGTAAGGCGCGGT)-3’,

primer 2: 5’-d(GTTTAAATCTCCCGGGGTTCGGA)-3’,

primer 3: 5’-d(GGGGTCCGGAGGGAGTCCAGAATCGA)-3’.

Cyclin A promoter:

primer 1:5’-d(AGCCAGGCCCTGCTA)-3’,

primer 2: 5’-d(CAGGCCGCCCAGTGCAGTCC)-3’,

primer 3: 5’-d(GCTCACCAGCTCGAGCCACGCAG)-3’

RESULTS

Micrococcal nuclease and methidiumpropyl-EDTA footprinting: evidence for a positioned nucleosome — Our first goal was to analyze the nucleosomal structure of the human cdc25C promoter between positions -290 and +121 which had previously been found to be necessary and sufficient for maximal activity and cell cycle regulation of the promoter (5). Towards this end, WI-38 fibroblasts were permeabilized, digested with increasing concentrations of MNase, and the digested DNAs were analyzed by LM-PCR using different cdc25C-specific primer sets. As shown in Figs. 1A and B, clusters of MNase hyperreactivity, which are indicative of nucleosomal linker regions, were seen
between positions -140 and ~-200 and between +8 and ~+50. In addition, less defined hyperreactive regions were identified upstream of position -280 (Fig. 1). The distance between the two proximal hyperreactive regions (148 bp) corresponded almost perfectly with the size of a nucleosomal core \textit{in vitro} (145 bp) (19, 20). In agreement with this observation, methidiumpropyl-EDTA (MPE) footprinting of the region between -200 and +50 revealed short hypersensitive stretches coinciding with the proximal MNase hyperreactive regions (Figs. 2A and B). These data strongly suggest that the proximal promoter region (-140 to +8), including the transcription factor binding sites necessary for activation and cell cycle specific repression, are organized around a positioned nucleosome (Fig. 3).

Since this region of the promoter is bound constitutively by the transcriptional activator NF-Y (6) and by the cell cycle-regulated transcriptional repressor CDF-1 (5, 9) we asked whether the nucleosomal structure might change during the cell cycle. We therefore performed MNase footprinting of synchronized cell populations, i.e. serum-deprived cells versus restimulated cells. However, these experiments did not show any cell cycle related differences with respect to the presence of hyperreactive nucleotides (data not shown). We therefore conclude that the proximal \textit{cdc25C} promoter region is organized as a positioned nucleosome and simultaneously occupied by transcription factors throughout the cell cycle.

\textit{DNase I footprinting the cdc25C promoter} — The chromatin structure is not only determined by histone acetylation but can also be influenced by transcription factor-induced remodeling of nucleosomes, as for example in the MMTV promoter (21). In this case, the binding of progesterone receptors leads to conformational changes within the
regulatory nucleosome, which in turn enables the interaction with other transcription factors and promoter activation (21).

In order to investigate the *cdc25C* promoter with respect to structural perturbations, bending or single-stranded stretches *in vivo* we performed genomic footprinting of the *cdc25C* promoter using different enzymatic or chemical conformation-sensitive probes. As shown in Fig. 4A, DNase I footprinting of permeabilized cells did not result in a characteristic 10bp pattern, which would be expected for rotationally positioned nucleosomal structures (22). Instead, protected areas were detected which coincided with the NF-Y and Sp1 binding sites previously identified by *in vivo* DMS footprinting (5). In agreement with these findings are the slight DNase I hyperreactivities 5´ and 3´of the Sp1 sites which are reported to occur adjacent to certain transcription factor binding sites *in vitro*. In addition, the close spacing of the NF-Y and Sp1 binding sites could contribute to the lack of a 10 bp pattern of DNase I hypersensitivity. Particularly strong hyperreactivities were seen between the NF-Y binding sites (arrows in Fig. 4A) suggesting that in these positions the minor groove of the double helix is exposed in a way that allows for a markedly preferred DNase I cleavage.

**Cell cycle dependence of DNase I protections and hyperreactivities in the cdc25C promoter** — Surprisingly, the DNaseI protections at the NF-Y sites were strongly reduced in resting cells (G₀) compared to normally cycling cells (growing; Fig. 4A). This is in apparent contrast to previously published *in vivo* DMS-footprinting data showing that the NF-Y binding sites are occupied *in vivo* throughout the cell cycle including G₀ cells (5). We attribute this to the fact that DNase I footprinting involves the permeabilisation of the cells by a detergent, while DMS treatment in carried with intact...
cells. It is possible that the interaction of NF-Y with its cognate site is weakened in G₀ cells, e.g. due to a decreased amount of NF-Y-A (23, 24), so that under the influence of a detergent this difference becomes detectable.

Another finding that deserves particular attention is the fact that the decreased protections at the NF-Y sites is associated with a reduction of the surrounding hyperreactivities (Figs. 4A and 5A). This suggests that a loss of NF-Y binding is correlated with a loss of hyperreactivity in vivo, and strongly supports the idea that NF-Y leads to drastic changes in the DNA structure upon binding to its cognate recognition site in vivo. This explanation is supported by similar observations made by OP-Cu footprinting of the cyclin A promoter as described below (see Fig. 5B).

**DNase I footprinting the cyclin A promoter** — To address the question as to whether such structural changes could also be observed in a structurally and functionally related, but different promoter we performed DNaseI footprints of the cyclin A gene which contains a single NF-Y side between a CDE-CHR module and an ATF site (Fig. 4B). Weak hyperreactivities could be detected surrounding the Sp1 site. The lack of hyperreactivities surrounding the NF-Y site which may be due to the close proximity of other transcription factor binding sites. In contrast, the occurrence of one strong hyperreactivity within the NF-Y binding site is striking. Thus, in the context of two different promoters NF-Y binding was associated with strong hyperreactivities (Fig. 4 A and B), suggesting a strong influence on DNA structure.

**Structural distortions in the cdc25C promoter detected by copper-phenanthroline footprinting** — Since NF-Y has been reported to bend DNA in vitro (16) we decided to
analyze the proximal promoter region for local distortions by copper-phenanthroline (OP-Cu) footprinting. OP-Cu is used to detect minor groove binding of transcription factors which sterically inhibit access to the C1-hydrogen or alter the DNA structure to a non-B-DNA conformation (25, 26). Both events result in OP-Cu hyporeactive or protected regions. OP-Cu can also be used to detect protein-induced conformational changes in the DNA which would lead to hyperreactive DNA stretches (27).

Even though OP-Cu had previously not been used for genomic footprinting, we were able to establish an appropriate procedure using permeabilized cells (see Materials and Methods for details). Thus, minor groove protections at the NF-Y binding sites and the CHR region were clearly detectable, whereas the sites of major groove binding (CDE, Sp1 binding sites) did not show any protections (Fig. 5 A). These expected results demonstrate the suitability of OP-Cu for genomic footprinting. Of particular interest, however, are the strong hyperreactivities between the NF-Y binding sites (arrows in Fig. 5 A). These indicate the presence of local distortions, which may be due to an unstacking of base pairs which creates more space for the intercalating phenanthroline moiety (28). Furthermore, the protections in the area of NF-Y binding are notably stronger compared to those in the CHR-region which is also occupied in the minor groove. This can be taken as further evidence for a non B-DNA structure at the NF-Y binding sites (25, 26, 28).

Copper-phenanthroline footprinting of the cyclin A promoter — For comparison we also footprinted the cyclin A promoter with OP-Cu (Fig 5 B). Protections were seen in the region of the NF-Y site and to a lesser extent at the CHR, as in the case of the cdc25C promoter, but also at the ATF site. Hyperreactivities were detected specifically
between the NF-Y site and the CDE/CHR element. No such hyperreactivity was found between the NF-Y and ATF sites (or other sites), indicating that the structural distortions detected by OP-Cu are indeed transcription factor-specific.

Cell cycle dependence of OP-Cu protections and hyperreactivities in the cyclin A promoter — We also analyzed potential cell cycle effects on the OP-Cu hyperreactivities in the cyclin A promoter. A comparison of the patterns obtained after footprinting of normally growing and G₀ cells showed a diminished protection of the NF-Y site and hyperreactivities 5′ to the NF-Y site in the G₀ cells (Fig 5B). These cell cycle effects are likely to reflect a weakened interaction of NF-Y with its cognate recognition site in G₀ cells and support the hypothesis that the OP-Cu hyperreactivities are caused by NF-Y binding in vivo. These observations are also consistent with the finding made with DNase I footprinting of the cdc25C promoter described above (Fig. 4A).

In contrast to the cdc25C promoter, the CDE was also protected, and hyperreactivities where detected in its vicinity. This presumably reflects the binding of additional factors of the E2F family with the CDE in the cyclin A promoter (7, 29, 30).

Structural distortions in the cdc25C promoter detected by KMnO₄ footprinting — Finally, we analyzed the proximal promoter region for kinked DNA structures or single stranded stretches by KMnO₄ footprinting in vivo. This study showed a strong correlation of KMnO₄ hyperreactivity with NF-Y binding (Fig. 6). Neither the Sp1 sites nor the CDE-CHR displayed such hyperreactivities. This KMnO₄ hyperreactivity, which coincides with the area of OP-Cu hypersensitivity, probably reflects local kinks or
strong bends with a defect in base stacking rather than a melted region, as has been reported for σ factor-induced DNA distortions in vitro (27). A strong DNA bend or unwinding with a local unstacking of the base pairs would enhance the intercalation of OP-Cu between the base pairs while giving KMnO₄ access to the 5,6-double bond of the T-ring (31).

Downstream of position +1 multiple sites of KMnO₄ hyperreactivity were also detectable, but these are presumably attributable to pausing polymerases in the basal promoter region (10).

**DISCUSSION**

The high resolution analysis of MNase and MPE hypersensitivities reported in the present study strongly suggests that the cdc25C promoter is organized in a positioned nucleosome, and that this structural organization is maintained throughout the cell cycle. The fact that the positioned nucleosome almost exactly spans the region encompassing the three NF-Y sites in the presence of bound NF-Y (5) strongly suggest that NF-Y interacts with a nucleosomal template in vivo. This is supported by the observation that NF-Y is capable of interacting with reconstituted nucleosomes in vitro (13).

The footprinting data also clearly demonstrate strong structural perturbations in and around the NF-Y binding sites in the context of two different promoters in vivo, i.e., cdc25C and cyclin A, that are structurally and functionally related (7). Of particular interest are the observed cell cycle effects, which indicate that NF-Y binding is decreased in resting cells, concomitantly with diminished hypersensitivities adjacent of
the NF-Y sites. These correlations also suggest that the observed structural perturbations in and around the NF-Y binding sites are indeed caused by NF-Y, which is in line with the ability of NF-Y to induce DNA-bending \textit{in vitro} (16).

The observed structural changes are not typical of rotationally positioned nucleosomes and rather point to a positioned but partially opened nucleosomal structure, as can also be seen in the MMTV promoter after hormone induction (21). It would be conceivable therefore that the structural disturbances at the NF-Y binding sites coinciding with NF-Y occupation of these sites, together with NF-Y’s reported ability to recruit the histone acetylases Gcn5 and P/CAF (11, 12), play a role in opening the chromatin structure of the proximal promoter. The OP-Cu and KMnO$_4$ footprints showing that NF-Y \textit{in vivo} obviously induces a local unstacking of the base pairs, which is indicative of kinked or strongly bend DNA, support this hypothesis, since the ability of transcription factors to bend DNA facilitates their binding to nucleosomal structures \textit{in vitro} (32). This is strongly supported by the data obtained with the cyclin A promoter, where a functionally crucial NF-Y site (7), albeit in a different context, is also associated with clear structural perturbations \textit{in vivo}.

The above observations lead to the following model: NF-Y acts as a transcriptional activator which may involve its property to recruit Gcn5 and P/CAF and its ability to bind to nucleosomal templates \textit{in vivo}. The structural perturbations at the NF-Y sites, reflecting changes in the nucleosomal structure caused by NF-Y may affect the interaction of the promoter with other factors, and may thus contribute to the transcriptional activation and/or repression of the gene. It might also be possible that the binding of the repressor CDF-1 in G$_0$/G$_1$ weakens the binding of NF-Y through an
unknown mechanism, which in turn results in an altered promoter topology that does not favor transcriptional activation. The data obtained in the present study provide the basis for future work addressing the validity of these ideas.

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FOOTNOTE

1The abbreviations used are: CBF: CAAT-box binding factor; CDE: cell cycle-dependent element; CDF-1: CDE-CHR binding factor-1; CHR: cell cycle genes homology region; DMS: dimethyl sulfate; DNase: deoxyribonuclease; DTT: dithioteitol; HDAC: histone deacetylase; LM-PCR: ligation-mediated polymerase chain reaction; MNase: micrococcus nuclease; MMTV: mouse mammary tumor virus; MPE: methidiumpropyl-EDTA; NF-Y nuclear factor-Y (CAAT-box binding factor); OP-Cu: phenanthroline copper; PCR: polymerase chain reaction; PMSF: phenylmethylsulfonyl fluoride.
FIGURE LEGENDS

Fig. 1. **MNase footprinting of the cdc25C promoter.** Normally growing WI-38 cells were permeabilized and treated with increasing amounts of MNase. For comparison genomic DNA was treated with MNase or DMS in vitro. The products were analyzed by LM-PCR using different specific sets. Analysis of the top strand (A) suggests the presence of a positioned nucleosome in the immediate 5′ region of the cdc25C promoter. More loosely positioned nucleosomes may be present further upstream. Analysis of the bottom strand (B) is in agreement with these results.

Fig. 2. **MPE footprinting of the cdc25C promoter.** Normally growing WI-38 cells were permeabilized, dounced and treated with increasing amounts of MPE. For comparison genomic DNA was treated with MPE or DMS in vitro. The products were analyzed by LM-PCR using primer set TS1. Analysis of the top strand shows short stretches of hyperreactive nucleotides between positions ~ -150 and -130 (A) and between positions +23 and +43 (B), respectively, suggesting the presence of a positioned nucleosome in the immediate 5′ region of the cdc25C promoter.

Fig. 3. **Overview of the nucleosomal structure of the cdc25C promoter.** The picture summarizes the in vivo MNase and MPE footprinting data for the cdc25C promoter region between positions -370 and +50. The rectangular boxes represent the protein-binding sites identified by genomic DMS footprinting (5). The arrows above the promoter indicate the MNase and MPE hyperreactivities identified by high resolution footprinting (Figs. 1 and 2). The positioning of a nucleosome deduced from these data is shown at the bottom.
Fig. 4. High resolution mapping of DNase I protections and hypersensitivities in the proximal human cdc25C (A) and cyclin A promoters (B). Normally growing WI-38 cells (panels A, B) and cells in G₀ (panel A) were permeabilized and analyzed by genomic DNase I footprinting followed by LM-PCR using the primer sets cdc25C TS1 or Cyclin A. For comparison genomic DNA was digested with DNase I or cleaved after DMS treatment in vitro. The hyperreactivities are marked by arrows.

Fig. 5. Genomic OP-Cu footprint of the proximal human cdc25C (A) and cyclin A promoters (B). Normally growing WI-38 cells (panels A, B) and cells in G₀ (panel B) were permeabilized and treated for different periods of time with the OP-Cu complex followed by LM-PCR using the primer sets cdc25C TS1 or Cyclin A. For comparison genomic DNA was treated with OP-Cu or DMS in vitro. The hyperreactivities are marked by arrows.

Fig. 6. High resolution mapping of KMnO₄-reactive nucleotides in the proximal human cdc25C promoter. Normally growing WI-38 cells were treated with increasing concentrations of KMnO₄. For comparison genomic DNA was treated with KMnO₄ and DMS in vitro. Structurally perturbed regions in the promoter (indicating kinked or melted DNA) are marked by arrows.
Figure 2

A.

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Figure 3