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The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21$^{WAF1/CIP1/SDI1}$ in S phase

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Cut is a homeodomain transcription factor which has the unusual property of containing several DNA-binding domains: three regions called Cut repeats and the Cut homeodomain. Genetic studies in *Drosophila melanogaster* indicate that *cut* plays important roles in the determination and maintenance of cell-type specificity. In the present study, we show that mammalian Cut proteins may yet play another biological role, specifically in proliferating cells. We found that the binding of Cut to a consensus binding site varies during the cell cycle. Binding was virtually undetectable in G_0 and early G_1, but became very strong as cells reached S phase. This was shown to result both from an increase in Cut expression and dephosphorylation of the Cut homeodomain by the Cdc25A phosphatase. We also show that the increase in Cut activity coincides with a decrease in p21$^{WAF1/CIP1/SDI1}$ mRNAs. In co-transfection experiments, Cut proteins repressed *p21$^{WAF1/CIP1/SDI1}$* gene expression through binding to a sequence that overlaps the TATA box. Moreover, *p21$^{WAF1/CIP1/SDI1}$* expression was repressed equally well by either Cdc25A or Cut. Altogether, these results suggest a model by which Cdc25A activates the Cut repressor which then downregulates transcription of p21$^{WAF1/CIP1/SDI1}$ in S phase. Thus, in addition to their role during cellular differentiation, Cut proteins also serve as cell-cycle-dependent transcriptional factors in proliferating cells.
Keywords: Cdc25A/CDP/cell cycle/Cut/p21$^{WAF1/CIP1/SDI1}$

Introduction

Genetic studies in *Drosophila melanogaster* have indicated that the *cut* locus plays an important role in determining cell-type specificity in several tissues (Bodmer *et al*., 1987; Blochlinger *et al*., 1990, 1991; Jack *et al*., 1991; Liu *et al*., 1991; Liu and Jack, 1992). The cDNAs for homologues of the *Drosophila* Cut homeodomain protein have recently been isolated from several mammalian species including human, dog, mouse and rat and were termed, respectively, CDP (CCAAT displacement protein), Clox (Cut-like homeobox), Cux (Cut homeobox) and CDP-2 (Andrés *et al*., 1992; Neufeld *et al*., 1992; Valarche *et al*., 1993; Dufort and Nepveu, 1994; Yoon and Chikaraishi, 1994). For simplicity, the terms human and mouse Cut (hCut and mCut), or Cut when describing Cut proteins in general, will be used in this article.

By analogy with other homeodomain proteins also conserved in evolution, it is assumed that Cut proteins play an equally important role in determining cell-type specificity in mammals (Andrés *et al*., 1992; Ludlow *et al*., 1996). In support of this notion, ectopic expression of Cut proteins from *D.melanogaster*, man or mouse was shown to have a similar effect on embryonic sensory organ development in *D.melanogaster* (Ludlow *et al*., 1996). Moreover, the 'cut wing' phenotype was rescued by all three proteins (Ludlow *et al*., 1996).

Sequence analysis of *cut* cDNA revealed that Cut is a homeodomain protein that contains three highly homologous regions of ~70 amino acids, the Cut repeats (Blochlinger *et al*., 1988). Cut repeats have been found to function as specific DNA binding domains, and the Cut repeat 3 was shown to cooperate with the Cut homeodomain (CR3HD) to bind to DNA with high affinity (Andres *et al*., 1994; Aufiero *et al*., 1994; Harada *et al*., 1994, 1995). Using PCR-mediated site selection and bacterially expressed Cut fusion proteins, a consensus binding site containing the ATCGAT sequence was identified (Andres *et al*., 1994; Aufiero *et al*., 1994; Harada *et al*., 1995). In addition, a certain number of the selected sequences diverged from this consensus, and yet were confirmed to be high-affinity recognition sites by electrophoretic mobility shift assay (EMSA) (Aufiero *et al*., 1994; Harada *et al*., 1995). Cut proteins were found generally to function as transcriptional repressors (Andres *et al*., 1992; Neufeld *et al*., 1992; Valarche *et al*., 1993; Dufort and Nepveu, 1994; Lievens *et al*., 1995; Mailly *et al*., 1996). Two active repression domains were identified within the C-terminal domain and Cut proteins were shown to downregulate gene expression via two mechanisms: competition for binding site occupancy and active repression (Mailly *et al*., 1996). In two independent studies, however, Cut proteins appeared to function as transcriptional activators and it was suggested that the regulatory effect of Cut on transcription may vary depending on the proteins with which it interacts (Yoon and Chikaraishi, 1994; Vanwijnen *et al*., 1996). In particular, supershift assays using antibodies against pRb-related proteins suggested that Cut may alternatively interact with pRb or p107 on different DNA sequences (Vanwijnen *et al*., 1996).

Progression through the cell cycle appears to be under the control of a complex regulatory circuitry involving cyclin-dependent kinases (CDK), CDK inhibitors (CKI) and the Cdc25 phosphatases (reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996; Dynlacht, 1997). One CDK inhibitor, p21$^{WAF1/CIP1/SDI1}$, was also found to inhibit the proliferating cell nuclear antigen (PCNA) whose

role is to confer processivity to DNA polymerase δ (Flores-Rozas *et al*., 1994; Waga *et al*., 1994). Thus, via these two biochemical activities, p21 can block cell-cycle progression in G_1. Several groups have demonstrated that $p21^{WAF1/CIP1/SDI1}$ mRNAs are downregulated to various extents at the end of G_1 and during S phase (Li *et al*., 1994; Michieli *et al*., 1994; Sheikh *et al*., 1994). Different transcription factors such as Sp1, Sp3, STAT, p53 and AP-2 have been shown to bind to the $p21^{WAF1/CIP1/SDI1}$ promoter and modulate its expression (el-Deiry *et al*., 1993; Datto *et al*., 1995; Biggs *et al*., 1996; Prowse *et al*., 1997; Zeng *et al*., 1997). However, the mechanism by which $p21^{WAF1/CIP1/SDI1}$ steady-state mRNA levels are decreased in S phase has not yet been elucidated.

The Cdc25 phosphatase was initially discovered in yeast as a mitosis promoting factor (Russell and Nurse, 1986). Three Cdc25 isoforms, A, B and C, have been discovered in mammalian cells. Cdc25C has been shown to fulfill in mammals a function similar to Cdc25 in yeast: it dephosphorylates cdc2 and activates the cdc2–cyclin B complex, allowing the G_2–M transition (Galaktionov and Beach, 1991; Strausfeld *et al*., 1991). Cdc25A on the other hand has been found to play a role in the G_1–S transition. It was found to become active in late G_1, to be activated by the cdk2–cyclin E complex and to block the inhibitory effect of $p21^{WAF1/CIP1/SDI1}$ on cyclin–cdk complexes (Hoffmann *et al*., 1994; Jinno *et al*., 1994; Saha *et al*., 1997). Moreover, microinjection of anti-Cdc25A antibodies in G_1 cells prevented progression into S phase (Hoffmann *et al*., 1994). In accordance with these results, Cdc25A expression was shown to be upregulated by c-Myc and it has been found to exhibit oncogenic properties (Galaktionov *et al*., 1995, 1996). Thus, Cdc25A plays an important role in the control of cell-cycle progression; however, apart from cdk2 and cdk4, its physiological targets remain unknown (Iavarone and Massague, 1997).

We have recently noted the absence of Cut DNA binding activity in quiescent cells following serum starvation (Coqueret *et al*.,1996). When cells were stimulated to re-enter the cell cycle, Cut DNA binding became detectable only several hours after serum addition, at a time which corresponded with the entry into S phase. These observations prompted us to investigate changes in Cut DNA binding activity between the G_1 and S phases, using various strategies to obtain populations of cells enriched in G_0, G_1 and S phases. We found that Cut DNA binding is very weak in G_0 and early G_1, whereas it is maximal in S phase. We investigated the basis for this modulation as well as its consequences at the molecular level. Our results reveal that Cut is part of a network which controls the G_1–S transition.

Results

Cut binds DNA at the end of the G_1 phase of the cell cycle

Using various mammalian cell lines, we have previously noticed that Cut DNA binding is very weak or undetectable in cells maintained in 0.4% serum, but is restored following the treatment of cell extracts with alkaline phosphatase (Coqueret *et al*., 1996). These results suggested that Cut DNA binding was inhibited by phosphorylation in G_0. In the present study, we set out to investigate Cut DNA binding activity during the cell cycle. The experimental approach we have taken was to synchronize fibroblastic cells using various strategies in order to obtain populations of cells enriched for a specific phase of the cell cycle: serum starvation–stimulation, double-thymidine block, treatment with hydroxyurea or mimosin (see Material and methods). Cell-cycle distribution was monitored by fluorescence activated cell sorting (FACS) analysis (Figures 1B, 2B and 3B) and Cut DNA binding was measured by EMSA using oligonucleotides encoding a consensus Cut DNA binding site (Figures 1A, 2A and 3A). Specific monoclonal antibodies were added to the binding reaction to confirm the presence of Cut proteins in the retarded protein–DNA complex (Figures 1A, lanes 8 and 9, and 2A, lanes 6 and 7). Cut DNA binding in NIH 3T3 cells was very weak in G_0 and early G_1 following serum starvation (Figures 1A, lanes 1–3, and 2A, lanes 2 and 3) and in G_1 after treatment with hydroxyurea or mimosin (Figure 2A, lanes 8 and 10). In contrast, Cut DNA binding was much stronger when cells were allowed to progress in the cell cycle (Figures 1A, lanes 4–6, and 2A, lanes 9 and 11–14; see also FACS in Figures 1B and 2B) or when cells were synchronized in G_1–S using the double-thymidine block procedure (Figure 2A, lanes 4 and 5). We conclude that whatever method of cell synchronization is used, Cut DNA binding is very weak in G_0 and early G_1 and is activated later in the cell cycle. Similar results were obtained using FR3T3 cells, rat embryo fibroblasts (REF) and 293T cells (Figure 3A; data not shown). Thus, in several cells Cut DNA binding activity is modulated during the cell cycle.

Interestingly, NIH 3T3 cells that were blocked in G_1 using mimosin did not progress in the cell cycle after release of the block, at least not for the first 8 h (Figure 2B, mimosin 0 and 8 h).Yet, Cut DNA binding was greatly increased during the same period (Figure 2A, lanes 11–14). These results suggested that the increase in Cut DNA binding may begin before cells have reached S phase, most likely in late G_1. Confirmation of this hypothesis was provided by the fact that in REF cells, Cut DNA binding started to increase 4 h following release of the mimosin block, while cells began to enter into S phase at 6 h (compare FACS and EMSA in Figure 3A, lanes 8–11, and B). We cannot exclude that the specific time at which Cut DNA binding activity is increased may depend on the method of cell synchronization, however, Cut DNA binding invariably is very weak in G_0 and early G_1 and is stronger when cells have passed the G_1–S transition.

Modulation of Cut DNA binding activity involves changes both in steady-state protein levels and phosphorylation status

Cut expression in populations of NIH 3T3 cells enriched in G_0, early G_1 and S was assessed by Western blot analysis using polyclonal antibodies raised against a recombinant human Cut protein (Figure 4A; Dufort and Nepveu, 1994). The corresponding FACS data were previously shown (Figure 2B, serum 0 h, serum 3 h, thymidine 0 h, thymidine 3 h). Two observations can be made. First, Cut proteins are detected in the four populations of cells. Thus, the near absence of Cut DNA binding in G_0 and early G_1 cannot be accounted for by the absence of Cut proteins. Secondly, Cut protein steady-state levels are increased in

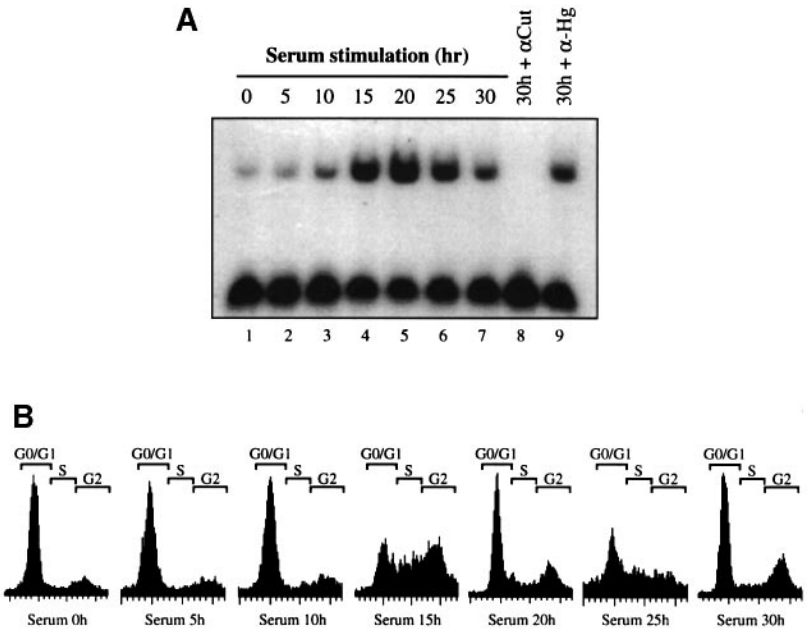

Fig. 1. Cut DNA binding activity is modulated during cell-cycle progression in NIH 3T3 cells. NIH 3T3 cells were synchronized in G_0 by serum starvation for 72 h and then stimulated by adding fresh Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The indicated time points correspond to the time elapsed since release from the G_0 block. Cut DNA binding activity was analyzed by EMSA and cell-cycle distribution was monitored by FACS analysis after staining of the DNA with propidium iodide. (**A**) EMSA: total extracts (5 μg of proteins) were incubated for 15 min at room temperature with radiolabeled oligonucleotides encoding a Cut consensus binding site in the presence or absence of monoclonal antibodies directed against either Cut (lane 8) or hemagglutinin (lane 9). DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**B**) FACS analyses.

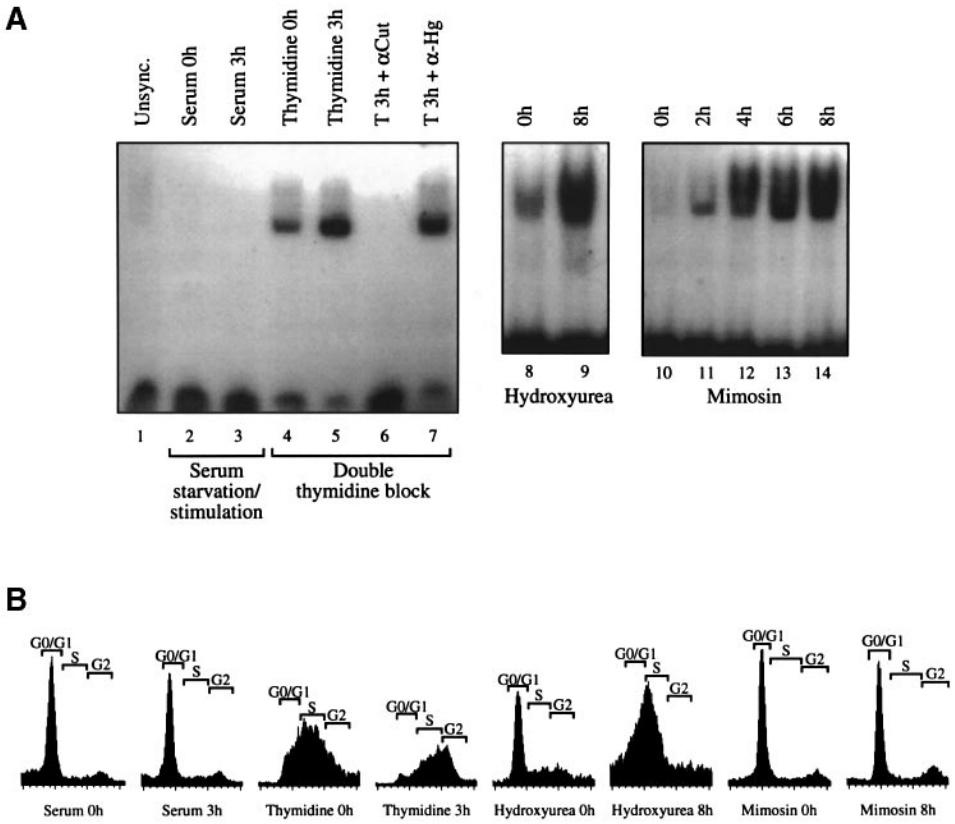

Fig. 2. Cell-cycle regulation of Cut DNA binding activity in NIH 3T3 cells. Populations of NIH 3T3 cells were enriched for different phases of the cell cycle using various cell synchronization methods as indicated (see Materials and methods). The indicated time points correspond to the time elapsed since release from the cell-cycle block. Cut DNA binding activity (**A**) and cell-cycle distribution (**B**) were analyzed as described in Figure 1. Unsync., unsynchronized cell population.

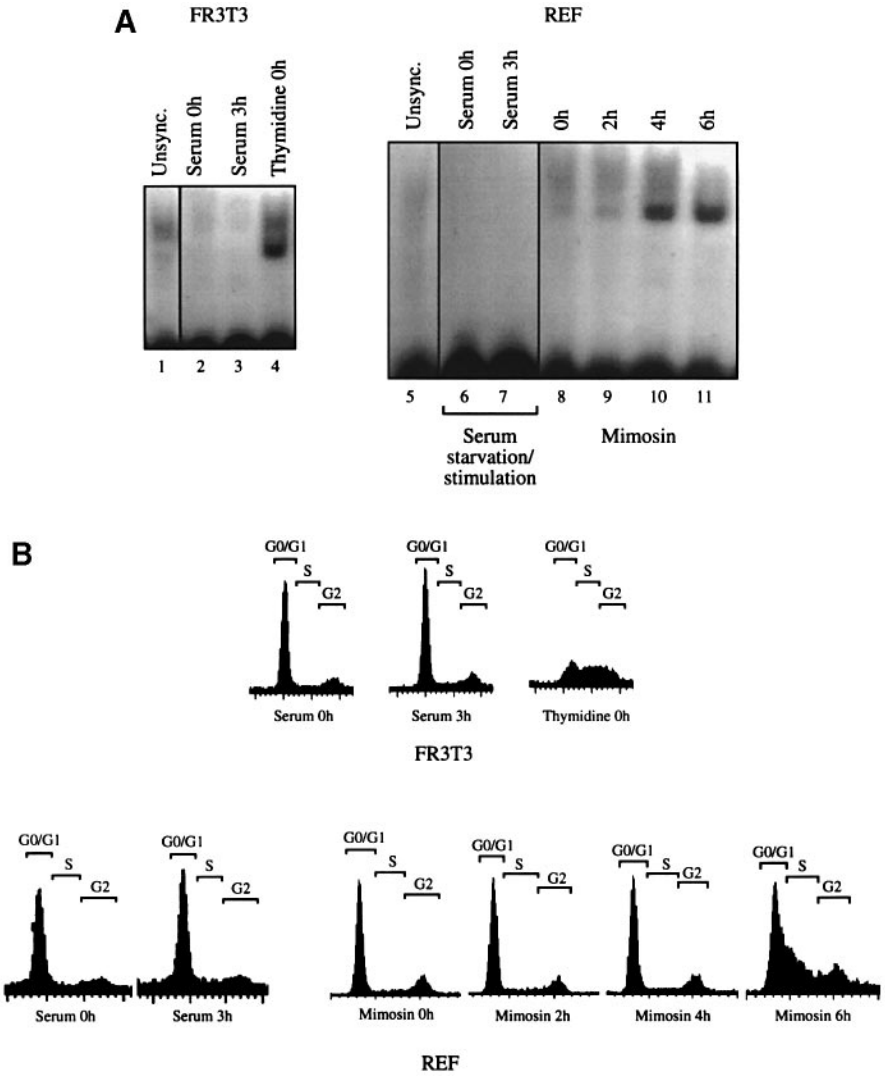

Fig. 3. Cut DNA binding activity is modulated during cell-cycle progression in FR3T3 and REF cells. Populations of FR3T3 and REF cells were enriched for different phases of the cell cycle using various cell synchronization methods as indicated (see Materials and methods). Cut DNA binding activity (**A**) and cell-cycle distribution (**B**) were analyzed as described in Figure 1.

the S phase-enriched population of cells and this probably contributes to the increase in Cut DNA binding (Figure 4A, lanes 3–4). Interestingly, Northern blot analysis indicated that the increase in Cut mRNA steady-state level preceded that of the proteins (Figure 4B).

We have previously demonstrated that the Cut DNA binding domains called the Cut repeats, can be phosphorylated by protein kinase C and casein kinase II, and that phosphorylation leads to an inhibition of DNA binding (Coqueret *et al*., 1996, 1998). We therefore hypothesized that modulation of Cut DNA binding during the cell cycle could occur, at least in part, at the level of phosphorylation. To verify this hypothesis, total cell extracts from populations of NIH 3T3 cells enriched in G_0, early G_1 and S were treated with calf intestinal phosphatase and then tested in EMSA (Figure 4C). Following dephosphorylation of the extracts, a significant increase in Cut DNA binding was observed in the G_0- and early G_1-extracts, but not in the S-extract. These results indicate that Cut DNA binding activity is inhibited by phosphorylation in G_0 and G_1 but not in S phase. Altogether, results from Western blot analysis and EMSA show that modulation of Cut DNA binding through the cell cycle involves two mechanisms: Cut protein expression is increased between G_1 and S and Cut DNA binding is inhibited by phosphorylation in G_0 and early G_1.

Phosphorylation of Cut was confirmed by *in vivo* labeling with [^{32}P]orthophosphate (Figure 4D) followed by immunoprecipitation and SDS–PAGE. Several bands with apparent mol. wt of ~200 kDa were detected and treatment with phosphatase decreased the intensity of the signal (Figure 4D, compare lanes 1–2 and 3–4).

Phosphorylation of CR3HD early in the cell cycle occurs within the Cut homeodomain and on serine residues

To compare Cut phosphorylation early and late in the cell cycle, cells were synchronized in early G_1 or in S phase prior to *in vivo* labeling. However, the phosphorylation status of Cut did not appear to be dramatically different (data not shown). One possibility we envisioned was that phosphorylation events in one part of the protein may mask changes in other domains. To investigate this further, we expressed a smaller portion of the Cut protein con-

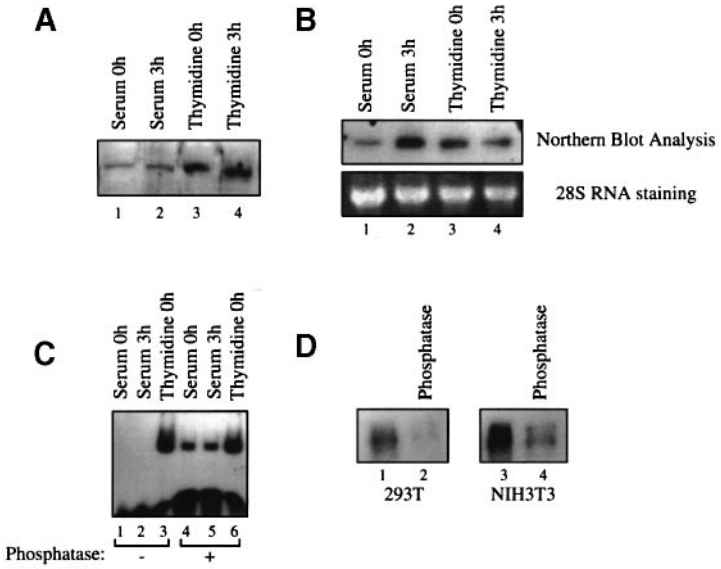

Fig. 4. Cut DNA binding activity is determined by protein expression level and by the phosphorylation status. (**A**) Western blot analysis showing increased Cut expression in S phase. NIH 3T3 cells were synchronized in G_0, early G_1, G_1–S and S phase as described in Figure 2, and Cut expression was monitored by Western blot analysis using 100 µg of proteins from total cell extracts and anti-Cut polyclonal antibodies. (**B**) Northern blot analysis showing Cut mRNA expression levels during the cell cycle. NIH 3T3 cells were synchronized in G_0, early G_1, G_1–S and S phase as described in Figure 2 and Cut mRNA expression was monitored by Northern blot analysis using 20 µg of total cellular RNA. Cut encodes a 5.5 kb mRNA, and the amount of RNA loaded was controlled by staining of the 28S RNA. (**C**) EMSA showing that Cut DNA binding activity in G_0 and early G_1 can be elevated by treatment of cell extracts with calf intestinal phosphatase. NIH 3T3 cells were synchronized in G_0, early G_1 and G_1–S phase as described in Figure 2. Total cell extracts (5 µg of proteins) were incubated for 30 min at room temperature in the presence (lanes 4–6) or absence (lanes 1–3) of calf intestinal phosphatase (30 mU/reaction). Radiolabeled oligonucleotides encoding a Cut binding site were added and after 15 min DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**D**) [^{32}P]orthophosphate *in vivo* labeling showing that Cut proteins are phosphorylated in NIH 3T3 and 293T cells. Unsynchronized (lanes 1–2) 293T cells or G_1-synchronized (lanes 3–4) NIH 3T3 cells were labeled overnight with [^{32}P]orthophosphate. Cell extracts were prepared and in some cases (lanes 2 and 4) incubated for 30 min with calf intestinal phosphatase. Following immunoprecipitation with polyclonal antibodies directed against Cut, proteins were separated by electrophoresis on 6% polyacrylamide gels and revealed by autoradiography.

taining only the DNA binding domain Cut repeat 3 and the homeodomain (CR3HD). On first attempts, the DNA binding activity of the exogenously expressed CR3HD protein was not found to be regulated in a cell-cycle-dependent manner. However, upon reducing the amount of transfected plasmid while increasing the proportion of transfected cells, we found conditions that would allow for the physiological modulation of CR3HD. As shown in Figure 5A, the DNA binding activity of CR3HD was much greater in S phase than in early G_1. Yet, the CR3HD protein was expressed at the same level in early G_1 and in S phase (Figure 5B). Importantly, following *in vivo* labeling with [^{32}P]orthophosphate, a much stronger signal was observed in early G_1 (Figure 5C). Taken together, these results indicate that as cells progress through the cell cycle, the CR3HD region becomes less phosphorylated and exhibits a higher DNA binding activity.

As mentioned earlier, we have previously shown that the phosphorylation of Cut repeats by PKC and CKII inhibits DNA binding. Thus, we postulated that phosphorylation at the PKC and CKII sites was responsible for the cell-cycle regulation of Cut DNA binding. To our surprise, however, a CR3HD molecule in which the PKC and CKII phosphorylation sites had been replaced with alanine, CR3HD^{S972A,S987A}, exhibited the same behavior as that of the wild-type (data not shown). Thus, the cell-cycle regulation of Cut DNA binding must involve other, as yet undefined, phosphorylation sites. As an initial step to identify such site(s), the 25 kDa CR3HD protein was purified by immunoprecipitation and SDS–PAGE and subjected to proteolysis digestion. Digestion with cyanogen bromide (CNBr) produced, in addition to the undigested fragment, one labeled fragment of ~12 kDa (Figure 5D). This fragment corresponded to the C-terminal portion of CR3HD. Therefore, phosphorylation must occur within or close to the homeodomain. Treatment of the gel with potassium hydroxide (KOH) eliminated the signal, suggesting that phosphorylation occurs on serine residues. Confirmation of this hypothesis was provided by acid hydrolysis: only one spot of phosphorylation was observed which comigrated with the phosphoserine control (Figure 5E). Altogether these results suggest that phosphorylation of a serine residue(s) within or close to the Cut homeodomain inhibits Cut DNA binding early in the cell cycle.

Cdc25A can associate with and dephosphorylate Cut

The activation of Cut DNA binding activity as cells approach S phase appears to involve two mechanisms: an increase in the steady-state level of Cut proteins and a reduction in the phosphorylation of the Cut homeodomain. Two observations suggest that this latter mechanism involves the action of a phosphatase. First, phosphatase treatment of cell extracts from S phase-enriched populations of cells did not further increase Cut DNA binding (Figure 4C). Thus, we can infer that the regulated serine residues within the Cut homeodomain exist essentially in an unphosphorylated state in S phase. Secondly, we have found that the Cut protein is very stable, with a half-life of $>$10 h. The pool of Cut protein in S phase must

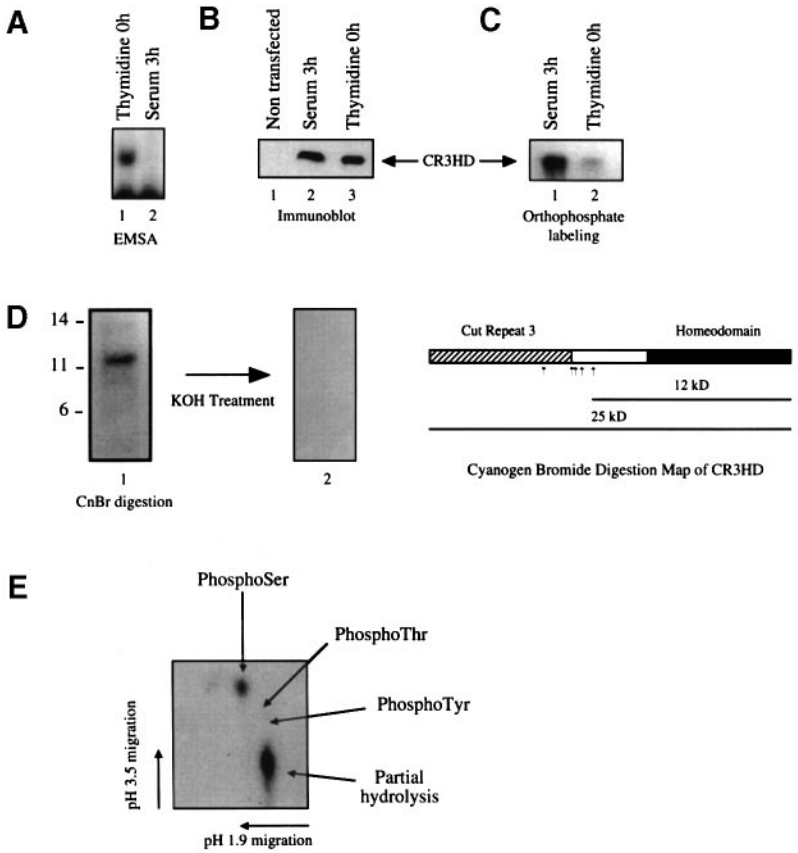

Fig. 5. Phosphorylation of CR3HD early in the cell cycle occurs within the Cut homeodomain and on serine residues. NIH 3T3 cells were transfected with either an empty vector (B, lane 1), or a vector expressing a histidine-tagged CR3HD [CR3HD–His; (A) lanes 1 and 2; (B) lanes 2 and 3; (C) lanes 1 and 2]. Cells were then synchronized in early G_1 [(A) lane 2; (B) lanes 1 and 2; (C) lane 1] or in G_1/S [(A) lane 1; (B) lane 3; (C) lane 2]. Protein expression level was analyzed by Western blot analysis (B) DNA binding activity by EMSA (A) and phosphorylation status by orthophosphate labeling and immunoprecipitation (C). (**A**) Total cell extracts (1 μg of proteins) were incubated for 15 min with radiolabeled oligonucleotides encoding a Cut binding site and DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**B**) One hundred micrograms of proteins from total cell extracts were subjected to Western blot analysis using anti-Cut polyclonal antibodies. (**C**) Transfected cells were synchronized in early G_1 (lane 1) or G_1–S phase (lane 2) as described in Figure 2 and incubated for 3 h in the presence of [^{32}P]orthophosphate. Cell extracts were prepared and CR3HD proteins were immunoprecipitated using anti-Cut polyclonal antibodies. Proteins were separated by electrophoresis on 15% SDS–polyacrylamide gel and revealed by autoradiography. (D and E) NIH 3T3 cells were transfected with a vector expressing CR3HD–His. Cells were then synchronized in early G_1 by serum starvation–stimulation and incubated for 3 h with [^{32}P]orthophosphate. CR3HD–His proteins were immunoprecipitated from total cell extracts using anti-Cut polyclonal antibodies, subjected to SDS–PAGE, transferred to PDVF membranes and visualized by autoradiography. The 25 kDa band was excised and subjected to proteolytic digestion. (**D**) CnBr digestion. Digested peptides were analyzed by electrophoresis on 18% polyacrylamide gels (lane 1). The membrane was then treated for 1 h at 60°C in 1 M KOH to remove serine and threonine residues, then washed with water and visualized again by autoradiography (lane 2). (**E**) Acid hydrolysis. The hydrolysate was applied to TLC plates and subjected to two-dimensional electrophoresis in pH 1.9 and 3.5 buffers. The mobilities of phosphoserine, phosphothreonine and phosphotyrosine are indicated according to the migration of a phosphoamino acid standard mixture.

therefore include proteins that were synthesized earlier, in early G_1, and were then dephosphorylated at the end of the G_1 phase. A few nuclear phosphatases have been found to be regulated in a cell-cycle-dependent manner. One of these, Cdc25A, was shown to become active at the end of G_1 and to be necessary for the G_1–S transition (Hoffmann *et al.*, 1994). We therefore asked whether Cdc25A could dephosphorylate Cut and, in doing so, activate its DNA binding activity. Transfection of a vector expressing Cdc25A lead to a significant increase in Cut DNA binding (Figure 6A). As a second approach to investigate whether Cdc25A may affect Cut DNA binding, cells were infected with a retroviral vector expressing Cdc25A under the control of a tetracycline-responsive tTA promoter. Expression of Cdc25A was induced upon removal of tetracycline from the medium and after 8 h, cell extracts were prepared and tested for Cut DNA binding. Again, Cut DNA binding was increased (Figure 6B, lanes 5 and 6), whereas no effect was noted when the same experiment was performed using a mutated Cdc25A molecule, Cdc25A^{C430S}, rendered inactive following the replacement of a cysteine with a serine residue within the catalytic domain (Figure 6B, lanes 3 and 4; Xu and Burke, 1996). These results demonstrate that overexpression of Cdc25A leads to the activation of Cut DNA binding. We then verified whether Cdc25A could interact with Cut. In a pull-down assay, Cut was specifically retained by a glutathione *S*-transferase (GST)–Cdc25A fusion protein but not by GST alone (Figure 6C, lanes 1 and 2). To ascertain the physiological significance of these results, we tested whether the interaction between Cut and Cdc25A could be revealed in co-immunoprecipitation experiments. For this assay, cells were transfected with a vector expressing a Cdc25A protein fused to a hemagglutinin (HA) tag (Cdc25–HA). Immunoprecipitation was performed alternatively with anti-HA, polyclonal or mono-

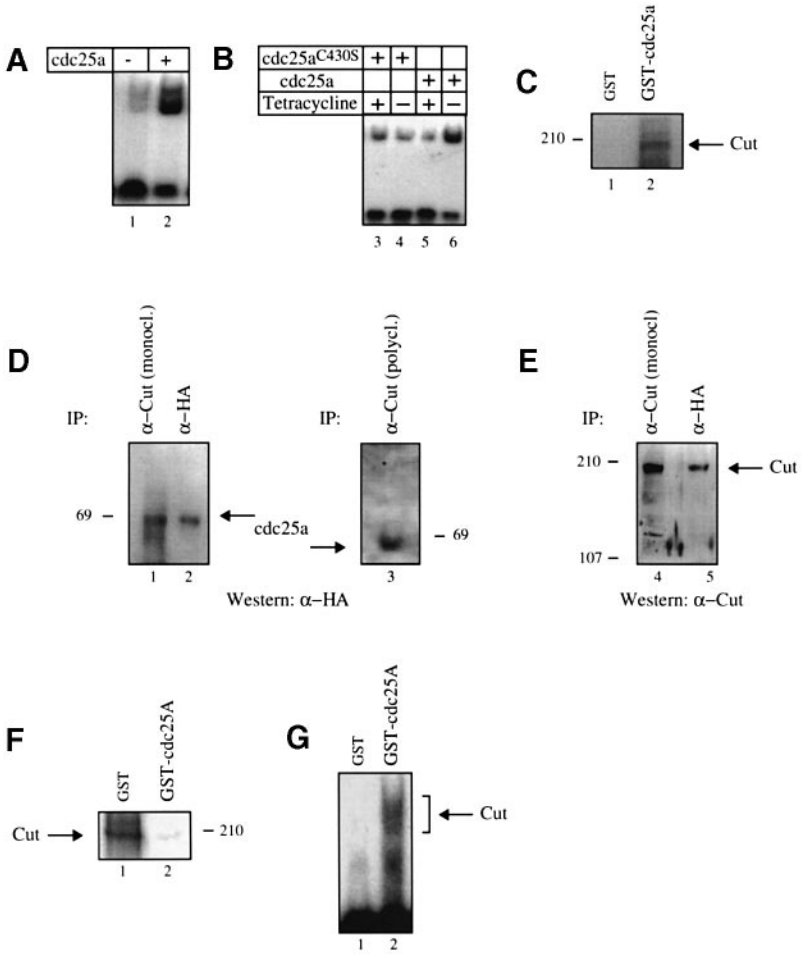

Fig. 6. Cut DNA binding activity is stimulated by Cdc25A. (**A**) NIH 3T3 cells were transfected with 2.5 μg of vector DNA expressing either Cdc25A (lane 2) or the parental vector (lane 1). Two days later, whole-cell extracts were prepared and tested in EMSA. Extracts (5 μg) were incubated for 15 minutes at room temperature with radiolabeled oligonucleotides encoding a Cut consensus binding site. DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**B**) NIH 3T3 cells were infected with retroviral vectors expressing either Cdc25A or $Cdc25A^{C430S}$ under the control of a tetracycline-responsive tTA promoter. Two days later, whole cell extracts were prepared and tested in EMSA as in (A). Where indicated (–), tetracycline was removed from the medium for 8 h prior to harvesting the cells in order to induce expression. (**C**) 293T total cell extracts (500 μg) were incubated for 1 h at 4°C with GST alone (lane 1) or GST–Cdc25A (lane 2) on glutathione beads. Samples were washed three times and separated on 6% SDS–polyacrylamide gels. Membranes were then blotted with anti-Cut antibodies. (D and E) 293T cells were transfected with a vector encoding the Cdc25A protein fused to a HA tag. Two days later, total cell extracts were prepared and used in immunoprecipitations. (**D**) Total cell extracts (1.5 mg) were immunoprecipitated using either monoclonal (lane 1) or polyclonal (lane 3) anti-Cut antibodies, or monoclonal anti-HA antibodies (lane 2). Immunoprecipitates (IP) were then separated on 10% polyacrylamide gels and immunoblotted with anti-HA antibodies. (**E**) Total cell extracts (5 mg) were immunoprecipitated using anti-HA antibodies (lane 5). Following immunoprecipitation, the supernatant was kept and one-fortieth (v/v) was loaded as a control to detect the proportion of Cut proteins which are not complexed with Cdc25A (lane 4). Samples were then separated on 6% polyacrylamide gels, and membranes were blotted with anti-Cut polyclonal antibodies. (**F**) NIH 3T3 cells were synchronized in G_0 by serum starvation for 72 h and labeled overnight with [^{32}P]orthophosphate in medium with 0.5% serum. Cell extracts were prepared and 5 mg of total cell extracts were immunoprecipitated using anti-Cut monoclonal antibodies. Immunoprecipitates were then incubated for 30 min at 30°C with GST alone (lane 1) or GST–cdc25A (lane 2). Proteins were separated by electrophoresis on 6% SDS–polyacrylamide gel and visualized by autoradiography. (**G**) NIH 3T3 cells were synchronized in G_0 by serum starvation for 72 h. Cell extracts were prepared and 5 mg of total cell extracts were immunoprecipitated using anti-Cut monoclonal antibodies. Immunoprecipitates were then incubated for 30 min at 30°C with GST alone (lane 1) or GST–cdc25A (lane 2), followed by a 20 min incubation on ice with deoxycholate 0.8%. The eluate was assayed for Cut DNA binding activity by EMSA.

clonal anti-Cut antibodies. Proteins present in the immunoprecipitates were revealed by immunoblotting with the reciprocal antibodies (Figure 6D and E). In each case, Cut and Cdc25A–HA were found to co-immunoprecipitate. It should be noted that co-immunoprecipitation with Cut was not observed with other HA-tagged proteins (data not shown), demonstrating the specificity of the Cut–Cdc25A interaction. To verify whether Cdc25A could directly dephosphorylate Cut and activate its DNA binding activity, we performed the following experiment. Cells were synchronized in G_0 by serum starvation for 72 h and radiolabeled or not with [^{32}P]orthophosphate in medium with 0.5% serum. Cut proteins were immunoprecipitated and incubated in the presence of GST or GST–Cdc25A. Proteins from radiolabeled cells were separated by SDS–PAGE and revealed by autoradiography (Figure 6F). Under these conditions, Cut was dephosphorylated by GST–Cdc25A but not by GST alone (Figure 6F). In parallel, proteins from unlabeled cells were further incubated in the presence of 0.8% deoxycholate and following centrifugation the eluates were tested in EMSA (Figure 6G). Cut DNA binding activity was increased only in the presence

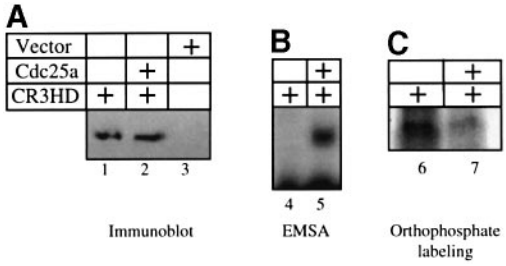

Fig. 7. Cdc25A dephosphorylates CR3HD. NIH 3T3 cells were transfected with the indicated expression vectors. Protein expression levels were analyzed by Western blot analysis (A), DNA binding activity by EMSA (B) and phosphorylation status by orthophosphate labeling and immunoprecipitation (C). (**A**) Proteins from total cell extracts (100 μg) were subjected to Western blot analysis using anti-Cut polyclonal antibodies. (**B**) Total cell extracts (1 μg of proteins) were incubated for 15 min with radiolabeled oligonucleotides encoding a Cut binding site and DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**C**) One day following transfection, cells were incubated overnight in the presence of [^{32}P]orthophosphate. Cell extracts were prepared and CR3HD proteins were immunoprecipitated using anti-Cut polyclonal antibodies. Proteins were separated by electrophoresis on 15% SDS–polyacrylamide gel and revealed by autoradiography.

of GST–Cdc25A. These results strongly suggest that Cdc25A acts directly on Cut; however, we cannot rule out the possibility that Cdc25A functions via another phosphatase which is immunoprecipitated with Cut.

We then investigated whether Cdc25A could also modulate the phosphorylation status and DNA binding activity of CR3HD. Following cotransfection in NIH 3T3 cells, the expression level of CR3HD was not affected (Figure 7A), whereas its phosphorylation was reduced (Figure 7C) and its DNA binding activity increased (Figure 7B). Thus, the CR3HD region contains all the elements necessary for the interaction with and modulation by Cdc25A. Altogether these results strongly suggest that Cdc25A can interact with Cut *in vivo* and dephosphorylate it.

The p21$^{WAF1/CIP1/SDI1}$ gene is downmodulated at the transcriptional level between the G_1 and S phases

We and others have previously demonstrated that Cut proteins function as transcriptional repressors. We therefore hypothesized that genes whose expression is downmodulated at the end of G_1 could be physiological targets of Cut. A survey of the literature revealed that only a few genes exhibit a decrease in mRNA steady-state levels between the G_1 and S phases. One of these is the *p21$^{WAF1/CIP1/SDI1}$* gene, whose product has been implicated in cell-cycle arrest in G_1 (Li *et al*., 1994; Michieli *et al*., 1994; Sheikh *et al*., 1994). We confirmed by Northern blot analysis that p21$^{WAF1/CIP1/SDI1}$ mRNA steady-state levels were decreased in a population of NIH 3T3 cells enriched in S phase (Figure 8A, lanes 1–4). To investigate whether downmodulation of p21$^{WAF1/CIP1/SDI1}$ mRNA levels occurs at a transcriptional level, we monitored the expression of a p21$^{WAF1/CIP1/SDI1}$/luciferase reporter construct, wwp–luc, in NIH 3T3 cells synchronized in early G_1 or S phase. Depending on the synchronization protocol (double-thymidine block or serum stimulation), luciferase activity was reduced 3- to 20-fold in S phase as compared to cells in early G_1 (Figure 8B). We conclude that downmodulation of p21$^{WAF1/CIP1/SDI1}$ mRNA between G_1 and S occurs, at least in part, at the transcriptional level.

Cut acts as a transcriptional repressor of the p21$^{WAF1/CIP1/SDI1}$ gene

Taken that the *p21$^{WAF1/CIP1/SDI1}$* gene is transcriptionally downmodulated in S phase and that Cut is a transcriptional repressor whose activity is highest in S phase, we hypothesized that Cut could repress *p21$^{WAF1/CIP1/SDI1}$*. To verify this, unsynchronized NIH 3T3 cells were cotransfected with the p21$^{WAF1/CIP1/SDI1}$/luciferase reporter construct, wwp–luc, and either an empty vector or a plasmid encoding the murine Cut protein. In six separate experiments, the luciferase activity was found to be reduced significantly in the presence of the Cut effector plasmid (Figure 9A, –2400/+70, $P < 0.05$).We then set out to identify the *cis*-acting elements within the p21$^{WAF1/CIP1/SDI1}$ promoter that are necessary for repression by Cut. First, we confirmed by S1 nuclease mapping analysis that the p21$^{WAF1/CIP1/SDI1}$ transcriptional start site corresponded to the 5′ end of the p21$^{WAF1/CIP1/SDI1}$ cDNA (Figure 8C). We then tested a series of reporter constructs with progressive 5′ and 3′ deletions (Figure 9A). The recombinant mCut protein significantly repressed each reporter construct with the exception of construct –61/+70 whose expression was too weak to allow a valid comparison (Figure 9A, $P < 0.05$, except –61/+70). Since deletion of 5′ flanking sequences up to –61 drastically reduced expression in NIH 3T3, this construct and a larger one in the series were tested in 293T cells. These two reporter plasmids were also repressed by mCut in 293T cells (Figure 9B, $P < 0.05$).

Altogether these results suggested that the p21$^{WAF1/CIP1/SDI1}$ proximal promoter is sufficient for repression by mCut (see DNA sequence in Figure 9G). Moreover, these results raised the possibility that the transcriptional downmodulation of *p21$^{WAF1/CIP1/SDI1}$* in S phase may be affected by the endogenous Cut protein. To verify this hypothesis, we first confirmed that the p21$^{WAF1/CIP1/SDI1}$ proximal promoter was downmodulated between G_1 and S (Figure 9C, $P < 0.05$).We then tested whether expression of Cut antisense RNA could relieve the repression of the same p21$^{WAF1/CIP1/SDI1}$/luciferase reporter construct in NIH 3T3 and 293T cells synchronized in S phase (Figure 9D and E). The –163/+70 p21$^{WAF1/CIP1/SDI1}$/luciferase reporter plasmid was introduced into NIH 3T3 and 293T cells together with a vector expressing antisense murine Cut RNA. Cells were then synchronized into S phase using the double-thymidine block procedure and luciferase activity was measured. Co-expression of Cut antisense RNA in each case lead to a 3-fold increase in luciferase activity (Figure 9D and E, $P < 0.05$). Western blot experiments confirmed that Cut protein steady-state levels were reduced in the presence of the Cut antisense vector (Figure 9F). We concluded that Cut protein is involved in the transcriptional downmodulation of *p21$^{WAF1/CIP1/SDI1}$* in S phase.

A sequence that overlaps the TATA box of the p21$^{WAF1/CIP1/SDI1}$ promoter is recognized by Cut

To localize Cut DNA binding site(s) within the p21$^{WAF1/CIP1/SDI1}$ core promoter, we performed DNase footprinting analysis using a bacterially expressed histidine-tagged fusion protein containing the Cut DNA binding domains Cut repeat 3 + homeodomain (CR3HD). Products

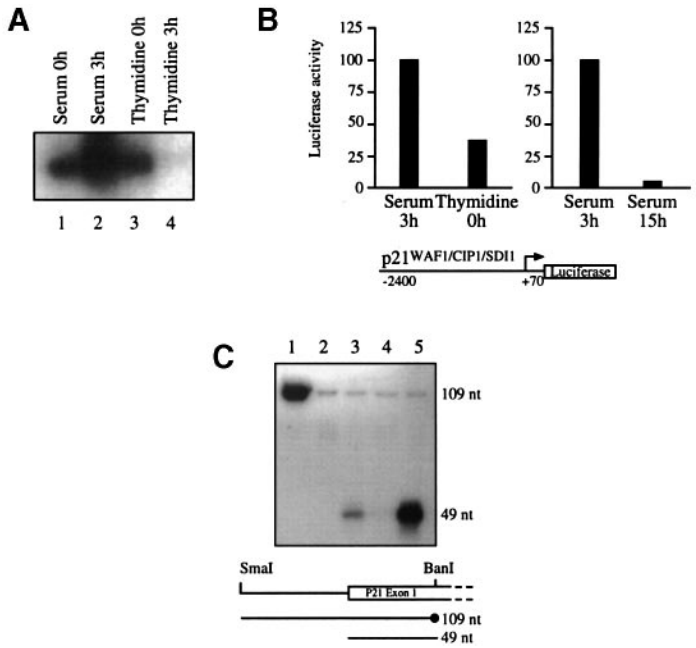

Fig. 8. Transcriptional downmodulation accounts, at least in part, for the decrease in p21$^{WAF1/CIP1/SDI1}$ mRNA levels in S phase. (**A**) Northern blot analysis of *p21$^{WAF1/CIP1/SDI1}$* expression in G_0, early G_1 and S phase. NIH 3T3 cells were synchronized in G_0, early G_1,G_1–S and S phase as described in Figure 2. Total RNA was prepared and 5 μg of RNA was subjected to Northern blot analysis using a murine p21$^{WAF1/CIP1/SDI1}$ cDNA probe. Equal RNA loading was verified by ethidium bromide staining or with hybridization of the membrane with a 18S ribosomal RNA probe (data not shown). (**B**) Luciferase transient transfection assay showing transcriptional downmodulation of the human p21$^{WAF1/CIP1/SDI1}$ promoter (wwp–luc; el-Deiry *et al*., 1993) in S phase. A p21$^{WAF1/CIP1/SDI1}$/luciferase reporter construct, wwp–luc, was introduced into NIH 3T3 cells which were then synchronized in early G_1 or in S phase. For early G_1, cells were maintained for 48 h in DMEM plus 0.4% FBS followed by a 3 h incubation in DMEM plus 10% FBS (Serum 3h). For S phase, quiescent cells were synchronized using the double-thymidine block procedure (Thymidine 0h) or stimulated with fresh DMEM with 10 % FBS for 15 h (Serum 15h). Cytoplasmic extracts were then prepared and processed to measure luciferase activity. Means of nine transfections are shown. Samples of cells were stained with propidium iodide and their DNA content assessed by FACS analysis (data not shown). (**C**) S1 nuclease mapping analysis of the p21$^{WAF1/CIP1/SDI1}$ transcription start site. A fragment of genomic DNA overlapping the 5′ end of the p21$^{WAF1/CIP1/SDI1}$ human cDNA was end-labeled at the *Ban*I site, cleaved at the *Sma*I site, hybridized with 20 μg of total RNA from various cells or tissues and treated with S1 nuclease. Lane 1, probe alone; lane 2, tRNA; lane 3, placenta; lane 4, 293T; lane 5, human mammary epithelial cells.

of a DNA sequencing reaction were run in parallel to identify the region(s) protected by CR3HD. We observed protection of a 22 bp region overlapping the TATA box and an Sp1 binding site (Figure 10A, lanes 5 and 6). We termed this region the Cut element (CE).

To verify whether endogenous Cut proteins can bind to the CE, EMSA were performed using 22 bp oligonucleotides encoding the CE sequence and extracts from NIH 3T3 cells enriched in early G_1 or in S phase. We observed a unique protein–DNA complex, which was stronger in S-phase extracts (Figure 10B, lanes 1 and 2) and disappeared when anti-Cut monoclonal antibodies were included in the reaction (Figure 10B, lanes 3 and 4). From these two criteria, we concluded that endogenous Cut proteins bind to the p21$^{WAF1/CIP1/SDI1}$ proximal promoter.

Insertion of the p21$^{WAF1/CIP1/SDI1}$ CE sequence into a heterologous promoter can confer sensitivity to repression by Cut

To verify whether the CE sequence from the p21$^{WAF1/CIP1/SDI1}$ promoter can mediate repression by Cut, we inserted four copies of the CE oligonucleotides into a heterologous promoter construct which, in previous studies, was found not to be repressed by Cut (Dufort and Nepveu, 1994). NIH 3T3 cells were cotransfected with the original MEC or the MEC4CE reporter construct in the presence or absence of a mCut effector plasmid. The MEC4CE reporter plasmid, but not the original MEC plasmid, was repressed by mCut (Figure 10C). These results demonstrate that the CE sequence from the p21$^{WAF1/CIP1/SDI1}$ promoter can mediate repression by Cut.

Overexpression of Cdc25A leads to the repression of p21$^{WAF1/CIP1/SDI1}$ transcription

Having shown that Cdc25A activates Cut DNA binding and that Cut represses the p21$^{WAF1/CIP1/SDI1}$ promoter, we then investigated whether Cdc25A could affect p21$^{WAF1/CIP1/SDI1}$ expression. Cells were cotransfected with the –163/+25 p21$^{WAF1/CIP1/SDI1}$/luciferase reporter plasmid and a vector expressing either Cut, Cdc25A or nothing (Figure 11). Luciferase activity was reduced in the presence of either Cut or Cdc25A. Thus, repression of the p21$^{WAF1/CIP1/SDI1}$ core promoter can be achieved alternatively by overexpressing Cut or Cdc25A. These results together with the evidence regarding the interaction of Cut and Cdc25A lead us to propose that repression of the p21$^{WAF1/CIP1/SDI1}$ core promoter by Cdc25A is mediated via dephosphorylation of Cut.

Discussion

Genetic studies in *D.melanogaster* demonstrated that *cut* plays an important role in determining cell-type specificity in several tissues. Since ectopic expression of the human and murine Cut proteins could complement certain *cut* mutants in *D.melanogaster*, it was proposed that the

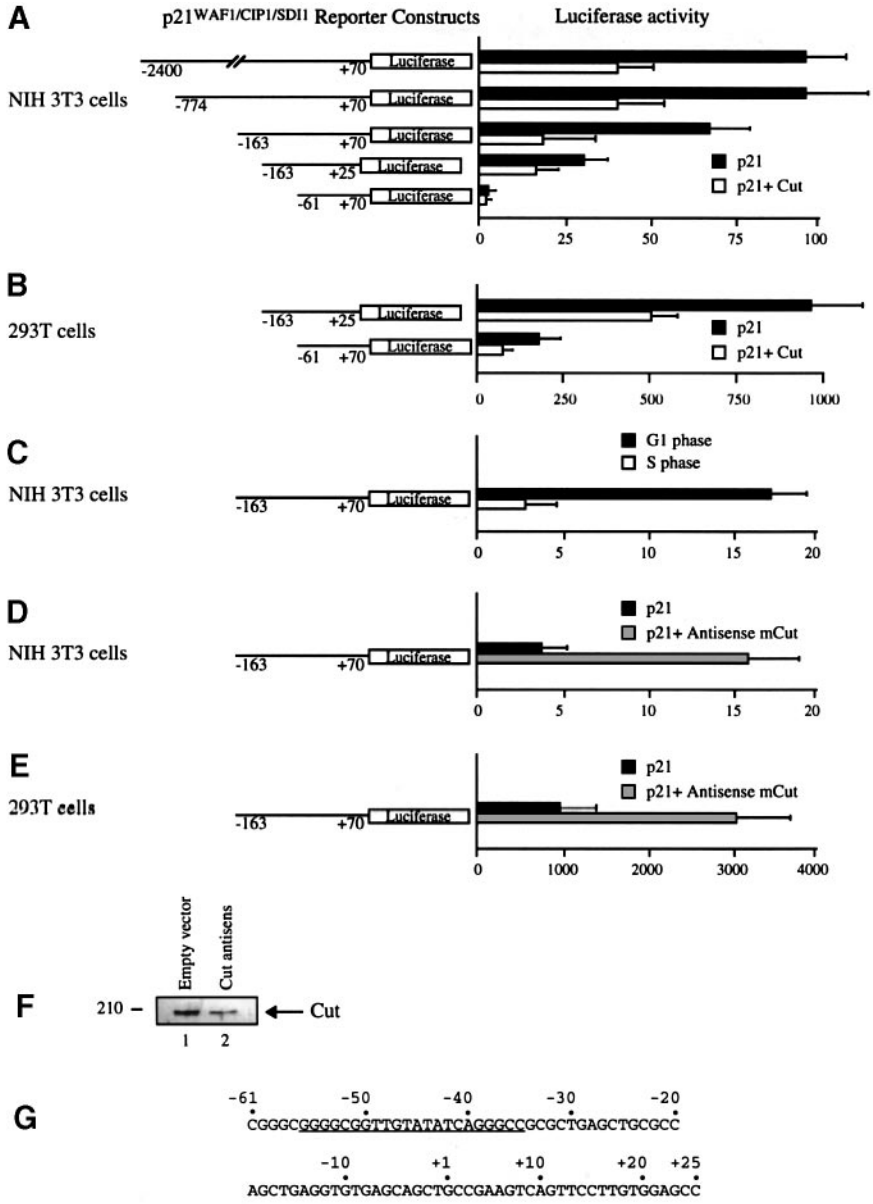

Fig. 9. Up- and downregulation of the $p21^{WAF1/CIP1/SDI1}$ promoter by sense and antisense Cut expression vectors. NIH 3T3 (**A**) and 293T (**B**) cells were cotransfected with various $p21^{WAF1/CIP1/SDI1}$/luciferase reporter constructs and either an empty vector or a Cut effector plasmid. Two days later, cytoplasmic extracts were then prepared and processed to measure luciferase activity. The mean of six transfections is shown. (**C**) NIH 3T3 cells were transfected with the –163/+70 $p21^{WAF1/CIP1/SDI1}$/luciferase reporter construct, and cells were synchronized using the double-thymidine block procedure. Cytoplasmic extracts were then prepared and processed to measure luciferase activity. The mean of six transfections is shown. Samples of cells were stained with propidium iodide and their DNA content assessed by FACS analysis (data not shown). NIH 3T3 (**D**) and 293T (**E**) cells were cotransfected with the –163/+70 $p21^{WAF1/CIP1/SDI1}$/luciferase reporter construct and either an empty vector or a Cut antisense vector. Cells were synchronized using the double-thymidine block procedure. Cytoplasmic extracts were then prepared and processed to measure luciferase activity. The mean of nine transfections is shown. Samples of cells were stained with propidium iodide andtheir DNA content assessed by FACS analysis (data not shown). (**F**) 293T cells were transfected with either an empty vector (lane 1) or a Cut antisense vector (lane 2). Two days later, Cut expression was monitored by Western blot analysis using 50 μg of proteins from total cell extracts and anti-Cut polyclonal antibodies. (**G**) The sequence of the $p21^{WAF1/CIP1/SDI1}$ promoter (from –61 to +25) which is sufficient for repression by Cut is presented. The underlined sequence represents the region protected by the Cut DNA binding domains in DNase footprinting analysis (see Figure 10).

function of *cut* is evolutionary conserved (Ludlow *et al*., 1996). On the basis of our results, we propose that Cut may play yet another biological role. First, Cut was found to be expressed in >30 different mammalian cell lines that were tested (data not shown). It thus seems that Cut is expressed in proliferating cells of many, if not all, types. Secondly, we have shown that Cut DNA binding activity is regulated in different phases of the cell cycle. Cut DNA binding activity is very weak in G_0 and early G_1, but much stronger later in the cell cycle. Thirdly, Cut was found to associate with and be activated by Cdc25A phosphatase, an important regulator of the G_1–S transition. Finally, evidence from transient transfection, EMSA and DNase footprinting studies suggested that Cut is involved in the regulation of $p21^{WAF1/CIP1/SDI1}$, the product of which is an important regulator of cell-cycle progression. Altogether, these results point to a novel biological role for Cut proteins. In addition to their role as determinants

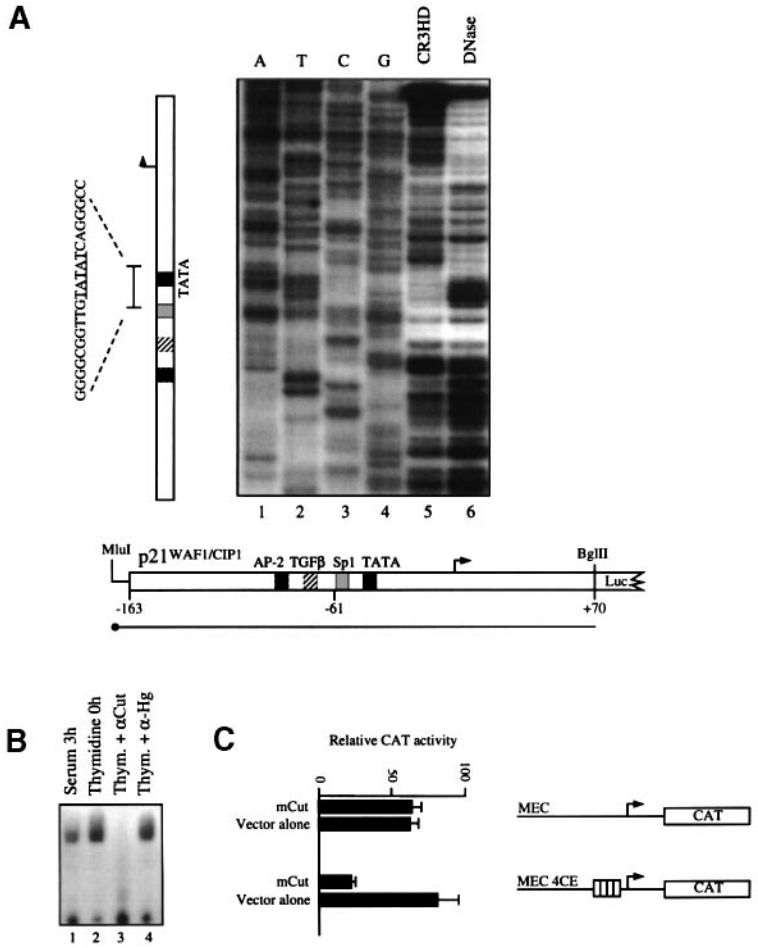

Fig. 10. A 22 bp region from the p21$^{WAF1/CIP1/SDI1}$ core promoter can mediate Cut DNA binding and repression activities. (**A**) DNase footprinting analysis of the interaction between Cut DNA binding domains (Cut repeat 3 + homeodomain) and the p21$^{WAF1/CIP1/SDI1}$ core promoter. The –163/+70 p21$^{WAF1/CIP1/SDI1}$/luciferase reporter construct was end-labeled at the *Mlu*I site and cleaved at the *Bgl*II site. The radiolabeled DNA fragment was incubated with 200 ng of purified bacterially expressed his/Cut repeat 3 + homeodomain (CR3HD) fusion protein prior to digestion with DNase I (lane 5). Lane 6 is a control lane where no protein was added. To identify the sequence that is protected by the CR3HD fusion protein, DNA sequencing was performed using as a primer an oligonucleotide whose 5′ end corresponds to that of the probe (lanes 1–4). The products of the DNase I and DNA sequencing reactions were run in parallel on a denaturing polyacrylamide gel. The protected sequence, nt –56/–35, is shown on the left. (**B**) EMSA showing interaction between the endogenous Cut protein and the p21$^{WAF1/CIP1/SDI1}$ core promoter. EMSA were performed using cellular extracts from NIH 3T3 cells synchronized either by the serum starvation–stimulation method (lane 1) or by the double-thymidine block procedure (lanes 2–4). Total extracts (5 μg of proteins) were incubated for 15 min at room temperature with radiolabeled oligonucleotides encoding the region of the p21$^{WAF1/CIP1/SDI1}$ core promoter that was protected in DNase footprinting assay (nt –56/–35, see underlined sequence in Figure 9). Where indicated, monoclonal antibodies directed against either Cut (lane 3) or hemagglutinin (lane 4) were added to the reaction. DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**C**) mCut can repress an heterologous promoter containing four copies of the Cut element from the p21$^{WAF1/CIP1/SDI1}$ promoter. NIH 3T3 cells were cotransfected with the MEC reporter construct containing or not containing four copies of the Cut element (CE, nucleotides –56/–35) from the p21$^{WAF1/CIP1/SDI1}$ promoter and either an empty vector or a Cut effector plasmid. Two days later, cytoplasmic extracts were prepared and processed to measure CAT activity.

of cell-type specificity during cellular differentiation, Cut proteins may also serve as cell-cycle-dependent transcriptional factors in proliferating cells.

An alternative hypothesis would be that Cut determines cell fate via its effect on cellular proliferation. In this model, cell-type specificity would be determined by the time at which a cell irreversibly exits the cell cycle, and by the specific cellular environment which prevails at that moment. Sustained Cut expression could promote further cell divisions at the expense of terminal differentiation. Cells that become terminally differentiated at a later time would do so in a slightly different environment and consequently may adopt a corresponding morphology. In contrast, the lack of Cut function in *cut* mutants would induce terminal differentiation at an earlier time, and consequently in a different environment. Although plausible, this model fails to explain the observation that maintenance of external sensory organ identity was contingent on the continued expression of Cut (Blochlinger *et al*., 1991). These results argue that Cut activity is not only necessary for proper differentiation, it is also essential of the maintenance of specific cell identity.

The above considerations lead us to favor the hypothesis that Cut fulfills at least two biological roles: one in proliferating cells and one in differentiated cells. We would then expect that Cut regulates different sets of genes in proliferating and differentiating cells. What then would be the basis for differential specificity of Cut's function? A few possibilities can be raised. First, four DNA binding domains have been identified within Cut: the three Cut repeats and the Cut homeodomain. Although the DNA binding properties of each domain has been

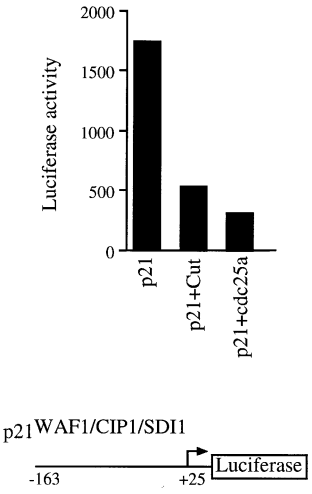

Fig. 11. Downregulation of the $p21^{WAF1/CIP1/SDI1}$ promoter by Cdc25A expression vector. 293T cells were cotransfected with the –163 to +25 $p21^{WAF1/CIP1/SDI1}$/luciferase reporter construct and a vector expressing either nothing (vector), Cut or Cdc25A. Two days later, cytoplasmic extracts were prepared and processed to measure luciferase activity.

investigated, it is not yet clear how the entire protein, with four DNA binding domains, interacts with DNA. One possibility is that not only Cut repeat 3 but also Cut repeat 1 or 2 has the potential to interact with the Cut homeodomain to form a bipartite DNA binding domain and that alternative arrangements of these domains may generate distinct DNA binding specificities. Alternatively, specificity may be conferred in part by Cut-interacting proteins. Such interactions would have the potential not only to affect DNA binding affinity but also the direction of Cut's effector activity. The effect of interacting proteins on effector function has already been detailed in the case of a few transcription factors that function either as activators or repressors depending on the context (Shi *et al*., 1991; Ayer *et al*., 1995; Kurokawa *et al*., 1995; Tini *et al*., 1995; Yang *et al*., 1996; Hurlin *et al*., 1997). In the case of Cut, it has been reported that it may associate with distinct Rb-related proteins on different promoters (Vanwijnen *et al*., 1996). It is thus possible that distinct Cut-containing complexes have opposite effects on transcription.

Our results have uncovered an important component of cell-cycle-dependent transcriptional regulation. Little is known on this subject apart for the induction of immediate-early genes, and the modulation of E2F/Dp complexes by the pocket proteins, Rb and its relatives p107 and p130. E2F/Dp complexes function as transcriptional activators, however, association with a pocket protein during G_0 and G_1 appears to convert them to repressors (reviewed in Luo *et al*., 1995; Muller, 1995; Whyte, 1995). Thus, an important molecular event in late G_1 is believed to be the phosphorylation of Rb proteins by CDKs, which causes the release of E2F and subsequent activation of several cell-cycle-regulated genes required for DNA replication. Another aspect of cell-cycle-dependent transcriptional regulation was recently uncovered. The periodic transcription of the cdc25C, cdc2 and cyclin A genes in G_2 was shown to be due to the periodic occupation of a repressor element, called the cell-cycle-dependent element (CDE) (Lucibello *et al*., 1995; Zwicker *et al*., 1995a,b; Liu *et al*., 1996). This element was bound in G_0 and G_1, but not in G_2, suggesting that upregulation of these genes in G_2 is allowed by the dissociation of a repressor from the CDE. Interestingly, the similarities between the promoters of these three genes is not limited to the presence of the CDE but also include the presence of several CCAAT and Sp1 binding sites within their upstream activating sequences. It was therefore proposed that CCAAT (NF-Y) and Sp1 binding proteins function as constitutive activators, whose activity is repressed in G_0 and G_1 through the CDE (Zwicker *et al*., 1995b). Some parallels can be drawn between the regulation of these genes and that of $p21^{WAF1/CIP1/SDI1}$. CCAAT and Sp1 binding sites are also present in the $p21^{WAF1/CIP1/SDI1}$ promoter and the presence of Sp1 sites was shown to be essential for expression (Datto *et al*., 1995). It is thus tempting to speculate that the periodic expression of $p21^{WAF1/CIP1/SDI1}$ during the cell cycle is regulated in an analogous manner to that of cdc25C. Thus, transcriptional downmodulation of $p21^{WAF1/CIP1/SDI1}$ in late G_1 and in S phase would be caused by the emergence of the Cut repression activity. Repression by Cut would probably involve two mechanisms, as we previously demonstrated: competition for binding site occupancy and active repression (Mailly *et al*., 1996).

The increase in Cut activity as cells reached S phase involved at least two mechanisms. The steady-state level of Cut proteins was higher in S phase and the phosphorylation state of the protein also appeared to vary such that DNA binding was almost completely inhibited by phosphorylation in G_0 and early G_1. In previous studies, we have shown that Cut repeats could be phosphorylated by protein kinase C (PKC) and casein kinase II (CKII) and that phosphorylation inhibited DNA binding (Coqueret *et al*., 1996, 1998). However, we have found that the levels of CKII and PKC activities do not vary between the G_1 and S phases (data not shown) and in agreement with these results, the site(s) of phosphorylation in G_0 was mapped within the Cut homeodomain, whose sequence does not contain any CKII nor PKC phosphorylation sites. Thus, the phosphorylation sites and the kinase(s) that modulates Cut DNA binding in G_0 and early G_1 remain to be identified; however, several results suggest that the flipped side of this regulatory switch involves the Cdc25A phosphatase. First, Cut and Cdc25A were found to be associated in pull-down and co-immunoprecipitation experiments. Secondly, overexpression of Cdc25A led to the activation of DNA binding by the endogenous Cut proteins. Thirdly, in cotransfection experiments, expression of a $p21^{WAF1/CIP1/SDI1}$ reporter plasmid was downmodulated to the same extent by either Cut or Cdc25A. These results lead us to propose a model by which Cdc25A activates the Cut repressor which then downregulates transcription of $p21^{WAF1/CIP1/SDI1}$. Interestingly, Cdc25A and $p21^{WAF1/CIP1/SDI1}$ have been shown to counterbalance each other by competing for cyclin–cdk complexes (Saha *et al*., 1997). Our results suggest a second mechanism by which Cdc25A could modulate the activity of $p21^{WAF1/CIP1/SDI1}$, that is, by activating a transcriptional repressor of the $p21^{WAF1/CIP1/SDI1}$ promoter.

Materials and methods

Cell culture and synchronization

NIH 3T3, FR3T3, REF and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

serum (FBS). To obtain cells in G_0 using the serum starvation–stimulation method, cells were maintained for 3 days in DMEM plus 0.4% FBS (Serum 0 h). The medium was then changed for DMEM plus 10% FBS and cells were harvested either 3 h later to obtain cells in early G_1 (Serum 3 h) or at later times after serum stimulation. Populations of cells enriched in G_1 were also obtained using mimosin and hydroxyurea. Mimosin: cells were maintained for 4 days in DMEM plus 0.4% FBS; the medium was then replaced for complete medium (DMEM plus 10% FBS) supplemented with 50 μM mimosin and cells were harvested 16 h later (Mosca *et al*., 1992). To allow cells to progress in the cell cycle, the medium was replaced with DMEM plus 10% FBS and cells were harvested at different times. Hydroxyurea: exponentially growing cells were incubated for 16 h in complete medium supplemented with 100 μM hydroxyurea (Herget *et al*., 1993). To allow cells to progress in the cell cycle, the medium was replaced with DMEM plus 10% FBS and cells were harvested at different times. Synchronization in G_1–S was performed using the double-thymidine procedure (Stein and Borun, 1972): cells were cultured overnight in DMEM plus 10% FBS supplemented with 2 mM thymidine, washed the next day, cultured for 10 h in DMEM plus 10% FBS and finally further incubated overnight in the presence of 2 mM thymidine (Thymidine 0 h). To allow cells to progress in the cell cycle, the medium was replaced with DMEM plus 10% FBS and cells were harvested 3 h later (Thymidine 3 h).

FACS analysis

To monitor cell-cycle distribution, cells were fixed, stained with propidium iodide and submitted to FACS analysis.

Transient transfections and preparation of cellular extracts

Cells were plated 24 h prior to transfection at a density of 0.3×10^6 per 100 mm plates for NIH 3T3 and 1×10^6 for 293T cells. All transfection experiments were repeated at least five times. Transient transfections were carried out using the calcium phosphate precipitation method. The amount of transfected DNA was kept constant by addition of appropriate amounts of the parental, empty, expression vector. After 48 h, cells were washed two times with PBS on ice and 200 μl buffer A [10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol (DTT)] was added to the plates. After 15 min incubation on ice, total extracts were recovered by centrifugation at 12 000 *g* for 10 min, and extracts were either used immediately or quick-frozen in a dry-ice ethanol bath and stored at –80°C.

In vivo labeling, peptide digestion and phosphoamino acid analysis

For [^{32}P]orthophosphate labeling, NIH 3T3 cells or 293T cells were rinsed once with phosphate-free DMEM containing 10% FBS and incubated for 5 h with the same medium. The DMEM was then replaced with the same medium containing 0.25 mCi/ml [^{32}P]orthophosphate in 5 ml and incubated for 3 h or overnight at 37°C (see figure legends). Cells were then washed two times with PBS on ice and 1 ml RIPA buffer (1.25% Nonidet P40, 1.25% sodium deoxycholate, 0.0125 M sodium phosphate pH 7.2, 2 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2 mM sodium vanadate and 100 mM sodium fluoride) was added to the plates. After 15 min incubation on ice, total extracts were recovered by centrifugation at 12 000 *g* for 10 min and subjected to immunoprecipitation using polyclonal antibodies directed against the C-terminal part of Cut. Proteins were then separated on 6% polyacrylamide gels. For CnBr digestion, CR3HD proteins were immunoprecipitated and electro-transferred to nylon membranes for at least 6 h at 4°C, the membrane was washed with water and the 25 kDa band corresponding to CR3HD was excised from the gel. The piece of membrane was then incubated for 1 h at 47°C with 30 mg/ml CnBr in 70% formic acid. The supernatant was dried using a Speed-Vac apparatus, washed in 100 μl of water and dried again. Dried peptides were then separated on 18% polyacrylamide gels and visualized by autoradiography. Phosphoamino acid analysis was performed by hydrolysis of the membrane-bound CR3HD proteins in constantly boiling 6N HCl for 1 h at 100°C. The resulting amino acids were dried, resuspended in 5 μl of pH 1.9 buffer (0.58 M formic acid, 1.36 M glacial acetic acid) and applied to TLC plates. Separation was achieved by two-dimensional electrophoresis, first in pH 1.9 buffer for 1 h at 500 V and then in pH 3.5 buffer [0.87 M glacial acetic acid, 0.5% pyridine (v/v), 0.5 mM EDTA] for 1 h at 500 V. One microliter of non-radioactive phosphoamino acid standards mixture containing phosphoserine, phosphothreonine and phosphotyrosine (10 mg/ml) were spotted on top of each sample. Following electrophoresis, the plate was dried for 20 min at 70°C and standards were visualized using 0.25% ninhydrin diluted in ethanol. Plates were then autoradiographed for 5 days at –80°C.

Retrovirus infection

The human Cdc25A construct (kindly provided by Dr D.Beach), was digested with *Eco*RI and *Bam*H1 to recover the Cdc25A fragment, which was then end-blunted and cloned into the *Not*1 site of the polylinker of the PBSTR1 retroviral vector (kindly provided by Dr S.A.Reeves). To produce retrovirus, 1×10^6 293T cells were transfected with 6 μg of the retroviral plasmid vector and 6 μg of helper DNA in the presence of tetracycline (10 ng/ml) using the calcium-phosphate procedure. Three days after the transfection, the medium containing the virus was removed, filtered and stored frozen until use. To investigate the effect of Cdc25A on Cut, NIH 3T3 plated at 15 000 cells/well (12-well plates) were infected with 1 ml of virus-containing supernatant in the presence of Polybrene (8 μg/ml) and tetracycline (10 ng/ml). Cells were washed the next day, incubated for 24 h in DMEM containing 10% FBS and tetracycline (10 ng/ml). Cdc25A expression was induced by removal of tetracycline for 8 h and cell extracts were then prepared as described above.

Electrophoretic mobility shift assays

Total extracts prepared as described above were preincubated for 5 min at room temperature in 25 mM NaCl, 10 mM Tris pH 7.5, 1 mM $MgCl_2$, 5 mM EDTA pH 8, 5% glycerol and 1 mM DTT with 0.5 μg of a PCR-amplified random oligonucleotide as a non-specific competitor. In selected experiments, extracts were preincubated for 30 min at room temperature with 30 mU of calf intestinal phosphatase and non-specific competitors were added for the last 5 min of reaction. A double-stranded nucleotide containing a Cut-consensus binding site (upper strand: 5′-AAAAGAA-GCTTATCGATACCGT-3′) was end-labeled using the Klenow polymerase and 10 pg of the probe (20 000 c.p.m.) was then incubated with the protein mixture for 15 min. Samples were then loaded on a 5% polyacrylamide gel (30:1) and separated by electrophoresis at 8 V/cm for 2 h in 50 mM Tris, 0.38 M glycine, 1 mM EDTA (pH 8.5). Gels were then dried and visualized by autoradiography.

$p21^{WAF1/CIP1/SDI1}$/luciferase reporter constructs

The human $p21^{WAF1/CIP1/SDI1}$ promoter construct, wwp–luc, a gift from Dr Vogelstein (el-Deiry *et al*., 1993), was used as a template for PCR-amplification of various portions of the promoter. Oligonucleotide primers (McGill Biotechnology Center) containing *Xba*I and *Bgl*II restriction sites were used for amplification. Following amplification, DNA fragments were digested with *Xba*I and *Bgl*II, purified and cloned into the *Nhe*I and *Bgl*II restrictions sites of the pGL3-Basic vector (Promega). The integrity of each vector was then verified by DNA sequencing. p21 –61/+70 was created by deleting the fragment of p21 –163/+70 between the *Sma*I site (base –63) and the *Sac*I site of the polylinker of pGL3. The plasmid was then treated with T4 DNA polymerase, purified on gel and ligated to itself.

Luciferase and chloramphenicol acetyltransferase (CAT) assays

Transfected cells were harvested with 1 ml of TEN buffer (40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl), centrifuged and resuspended in 100 μl of KH_2PO_4 0.1 M (pH 7.8), 0.1% Triton, incubated for 10 min on ice and then centrifuged for 10 min. Extracts were then used to measure luciferase activity by integrating total light emission over 10 s using a LKB 1250 luminometer. The luciferase activity was normalized based on protein concentrations. For CAT analysis, cell pellets were resuspended in 100 μl of Tris 0.25 M (pH 7.5) and incubated for 10 min on ice. Cells were then subjected to three cycles of freeze–thaw, and following centrifugation, cellular extracts were recovered and used directly in CAT assays or stored frozen at –80°C. CAT assays were performed as described and visualized by autoradiography (Gorman *et al*., 1982).

Western blot and immunoprecipitation analysis

Total protein extracts were recovered as described above and lysates (1–5 mg) were precleared for 1 h by incubation with a pre-immune serum followed by incubation for 1 h with 40 μl of protein A–Sepharose. Immunoprecipitations (1 ml) were then performed with the indicated antibodies for 1.5 h on a rotator and 30 μl of protein A–Sepharose was added and incubated for 45 min at 4°C. Immunoprecipitates were washed three times with lysis buffer and resuspended in 2× SDS sample buffer. For Western blot, total protein extracts were recovered as described above, centrifuged and 100 μg were resuspended in 40 μl of Laemmli

buffer. Protein were then boiled for 5 min and loaded on SDS–polyacrylamide gels. The gel was soaked for 10 min in 0.1 M Tris, 0.192 M glycine, 20% (v/v) methanol, and proteins were electro-transferred to nylon membranes for at least 6 h at 4°C. Blots were then washed five times with TBS (10 mM Tris pH 8, 150 mM NaCl) supplemented with 0.5% Tween (TBS–Tween 0.1%), and incubated overnight at 4°C in TBS containing 5% milk and 2.5% bovine serum albumin (BSA) to prevent non-specific binding of the antibody. Following five washings with TBS–Tween 0.5%, blots were then incubated with indicated antibodies diluted in TBS buffer (5% milk, 2.5% BSA) (Dufort and Nepveu, 1994). After washings, membranes were incubated with a second antibody conjugated to horseradish peroxidase for 40 min at room temperature and washed five times in TBS–Tween 0.5%, one time in TBS–Tween 0.1% and once in TBS. Proteins were then visualized using the ECL system of Amersham according to the manufacturer's instructions.

Northern blot analysis

Northern blot analysis was performed essentially as described previously (Thomas, 1980). Total RNA was size fractionated on a denaturing 6% formaldehyde–1% agarose gel and transferred to nitrocellulose (Schleicher and Schuell). After 4 h of prehybridization, hybridization was carried out overnight at 42°C in 5 ml of 50 mM HEPES (pH 7), 0.75 M NaCl, 50% formamide, 3.5% SDS, 5× Denhart's, 2 mM EDTA, 0.1% SDS and 200 μg/ml salmon sperm DNA. The full length p21 or Cut mouse cDNA were labeled with [^{32}P]dCTP using the random-priming labeling kit from Gibco-BRL (specific activity $>10^9$ c.p.m./μg) and were used as a probe. Filters were then washed three times in 0.1× SSC, 0.1% SDS, at 65°C for 20 min each. They were then exposed to X-ray film with intensifying screens at –80°C overnight.

DNase footprinting analysis

The p21 –163/+70 construct was used for this analysis. The plasmid was ^{32}P-labeled at the *Mlu*I site within the polylinker with T4 polynucleotide kinase and cleaved with *Bgl*II. After electrophoresis through a 5% polyacrylamide gel, the labeled fragments were purified by passive elution in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA. DNase footprinting analysis was performed as previously described (Harada *et al.*, 1995). End-labeled DNA (8000 c.p.m. per tube) was incubated with 200 ng of bacterially expressed fusion proteins purified by affinity chromatography for 15 min at room temperature in a final volume of 75 μl in 10 mM Tris (pH 7.5), 25 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 4% (w/v) polyvinyl alcohol. Two hundred and twenty-five microliters of 10 mM $MgCl_2$, 5 mM $CaCl_2$ was added for 90 s. Various dilutions of DNase I were added and samples were then incubated for 90 s. Two hundred and seventy microliters of DNase stop solution (20 mM EDTA, 1% SDS, 0.2 M NaCl) were then added and mixed by vortexing. Following phenol–chloroform extraction and ethanol precipitation, samples were electrophoresed through a 8% denaturing polyacrylamide (40:1) gel in 1× Tris borate–EDTA. Gels were dried and visualized by autoradiography.

S1 mapping analysis

S1 nuclease protection analysis was performed essentially as previously described (Yang *et al.*, 1986). DNA probes were end-labeled using T4 kinase (Maxam and Gilbert, 1980). Fifty micrograms of cytoplasmic RNA was annealed to 2×10^5 c.p.m. of end-labeled probe, at 55°C for 8 h, in 80% formamide, 0.4 M NaCl, 0.4 M PIPES (pH 6.4), 1 mM EDTA. RNA–DNA hybrids were digested with 500 units of S1 nuclease (Gibco-BRL) at 25°C for 45 min and at 37°C for 15 min. Following phenol–chloroform extraction and ethanol precipitation, RNA–DNA hybrids were digested with 500 units of S1 Nuclease (Gibco-BRL) for 60 min at 25°C and separated by electrophoresis on a 8 M urea, 4% polyacrylamide gel.

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References

Andres,V., Nadal-Ginard,B. and Mahdavi,V. (1992) Clox, a mammalian homeobox gene related to *Drosophila cut*, encodes DNA-binding regulatory proteins differentially expressed during development. *Development*, **116**, 321–334.

Andres,V., Chiara,M.D. and Mahdavi,V. (1994) A new bipartiteDNA-binding domain: cooperative interaction between the cut repeat and homeo domain of the cut homeo proteins. *Genes Dev.*, **8**, 245–257.

Aufiero,B., Neufeld,E.J. and Orkin,S.H. (1994) Sequence-specific DNA binding of individual Cut repeats of the human CCAAT displacement/Cut homeodomain protein. *Proc. Natl Acad. Sci. USA*, **91**, 7757–7761.

Ayer,D.E., Lawrence,Q.A. and Eisenman,R.N. (1995) Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell*, **80**, 767–776.

Biggs,J.R., Kudlow,J.E. and Kraft,A.S. (1996) The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells. *J. Biol. Chem.*, **271**, 901–906.

Blochlinger,K., Bodmer,R., Jack,J., Jan,L.Y. and Jan,Y.N. (1988) Primary structure and expression of a product from Cut, a locus involved in specifying sensory organ identify in *Drosophila*. *Nature*, **333**, 629–635.

Blochlinger,K., Bodmer,R., Jack,J.W., Jan,L.Y. and Jan,Y.N. (1990) Patterns of expression of Cut, a protein required for external sensory organ development in wild-type and cut mutant *Drosophila* embryos. *Genes Dev.*, **4**, 1322–1331.

Blochlinger,K., Jan,L.Y. and Jan,Y.N. (1991) Transformation of sensory organ identity by ectopic expression of Cut in *Drosophila*. *Genes Dev.*, **5**, 1124–1135.

Bodmer,R., Barbel,S., Shepherd,S., Jack,J.W., Jan,L.Y. and Jan,Y.N. (1987) Transformation of sensory organs by mutations of the cut locus of *D.melanogaster*. *Cell*, **51**, 293–307.

Coqueret,O., Berube,G. and Nepveu,A. (1996) DNA binding by cut homeodomain proteins is down-modulated by protein kinase C. *J. Biol. Chem.*, **271**, 24862–24868.

Coqueret,O., Martin,N., Berube,G., Rabat,M., Litchfield,D. and Nepveu,A. (1998) DNA binding by cut homeodomain proteins is down-modulated by casein kinase II. *J. Biol. Chem.*, **273**, 2561–2566.

Datto,M.B., Yu,Y. and Wang,X.F. (1995) Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. *J. Biol. Chem.*, **270**, 28623–28628.

Dufort,D. and Nepveu,A. (1994) The human cut homeodomain protein represses transcription from the c-myc promoter. *Mol. Cell. Biol.*, **14**, 4251–4257.

Dynlacht,B.D. (1997) Regulation of transcription by proteins that control the cell cycle. *Nature*, **389**, 149–152.

el-Deiry,W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Flores-Rozas,H., Kelman,Z., Dean,F.B., Pan,Z.Q., Harper,J.W., Elledge,S.J., O'Donnell,M. and Hurwitz,J. (1994) Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc. Natl Acad. Sci. USA*, **91**, 8655–8659.

Galaktionov,K. and Beach,D. (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, **67**, 1181–1194.

Galaktionov,K., Lee,A.K., Eckstein,J., Draetta,G., Meckler,J., Loda,M. and Beach,D. (1995) CDC25 phosphatases as potential human oncogenes. *Science*, **269**, 1575–1577.

Galaktionov,K., Chen,X. and Beach,D. (1996) Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature*, **382**, 511–517.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.*, **2**, 1044–1051.

Harada,R., Dufort,D., Denis-Larose,C. and Nepveu,A. (1994) Conserved cut repeats in the human cut homeodomain protein function as DNA binding domains. *J. Biol. Chem.*, **269**, 2062–2067.

Harada,R., Berube,G., Tamplin,O.J., Denis-Larose,C. and Nepveu,A. (1995) DNA-binding specificity of the cut repeats from the human cut-like protein. *Mol. Cell. Biol.*, **15**, 129–140.

Harper,J.W. and Elledge,S.J. (1996) Cdk inhibitors in development and cancer. *Curr. Opin. Gen. Dev.*, **6**, 56–64.

Herget,T., Brooks,S.F., Broad,S. and Rozengurt,E. (1993) Expression of the major protein kinase C substrate, the acidic 80-kilodalton myristoylated alanine-rich C kinase substrate, increases sharply when Swiss 3T3 cells move out of cycle and enter G_0. *Proc. Natl Acad. Sci. USA*, **90**, 2945–2949.

Hoffmann,I., Draetta,G. and Karsenti,E. (1994) Activation of the phosphatase activity of human cdc25A by a cdk2–cyclin E dependent phosphorylation at the G_1/S transition. *EMBO J.*, **13**, 4302–4310.

Hurlin,P.J., Queva,C. and Eisenman,R.N. (1997) Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev.*, **11**, 44–58.

Iavarone,A. and Massague,J. (1997) Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature*, **387**, 417–422.

Jack,J., Dorsett,D., Delotto,Y. and Liu,S. (1991) Expression of the Cut locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. *Development*, **113**, 735–747.

Jinno,S., Suto,K., Nagata,A., Igarashi,M., Kanaoka,Y., Nojima,H. and Okayama,H. (1994) Cdc25A is a novel phosphatase functioning early in the cell cycle. *EMBO J.*, **13**, 1549–1556.

Kurokawa,R., Soderstrom,M., Horlein,A., Halachmi,S., Brown,M., Rosenfeld,M.G. and Glass,C.K. (1995) Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature*, **377**, 451–454.

Li,Y., Jenkins,C.W., Nichols,M.A. and Xiong,Y. (1994) Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene*, **9**, 2261–2268.

Lievens,P.M.J., Donady,J.J., Tufarelli,C. and Neufeld,E.J. (1995) Repressor activity of CCAAT displacement protein in HL-60 myeloid leukemia cells. *J. Biol. Chem.*, **270**, 12745–12750.

Liu,S. and Jack,J. (1992) Regulatory interactions and role in cell type specification of the Malpighian tubules by the Cut, Kruppel and caudal genes of *Drosophila*. *Dev. Biol.*, **150**, 133–143.

Liu,S., McLeod,E. and Jack,J. (1991) Four distinct regulatory regions of the Cut locus and their effect on cell type specification in *Drosophila*. *Genetics*, **127**, 151–159.

Liu,N.S., Lucibello,F.C., Zwicker,J., Engeland,K. and Muller,R. (1996) Cell cycle-regulated repression of B-Myb transcription—cooperation of an E2F site with a contiguous corepressor element. *Nucleic Acids Res.*, **24**, 2905–2910.

Lucibello,F.C., Truss,M., Zwicker,J., Ehlert,F., Beato,M. and Muller,R. (1995) Periodic cdc25C transcription is mediated by a novel cell cycle-regulated repressor element (CDE). *EMBO J.*, **14**, 132–142.

Ludlow,C., Choy,R. and Blochlinger,K. (1996) Functional analysis of *Drosophila* and mammalian Cut proteins in flies. *Dev. Biol.*, **178**, 149–159.

Luo,Y., Hurwitz,J. and Massague,J. (1995) Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature*, **375**, 159–161.

Mailly,F., Berube,G., Harada,R., Mao,P.L., Phillips,S. and Nepveu,A. (1996) The human Cut homeodomain protein can repress gene expression by two distinct mechanisms—active repression and competition for binding site occupancy. *Mol. Cell. Biol.*, **16**, 5346–5357.

Maxam,A. and Gilbert,W. (1980) Sequencing end-labelled DNA with base-specific chemical cleavage. *Methods Enzymol.*, **65**, 499–580.

Michieli,P., Chedid,M., Lin,D., Pierce,J.H., Mercer,W.E. and Givol,D. (1994) Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.*, **54**, 3391–3395.

Mosca,P.J., Dijkwel,P.A. and Hamlin,J.L. (1992) The plant amino acid mimosine may inhibit initiation at origins of replication in Chinese hamster cells. *Mol. Cell. Biol.*, **12**, 4375–4383.

Muller,R. (1995) Transcriptional regulation during the mammalian cell cycle. *Trends Genet.*, **11**, 173–178.

Neufeld,E.J., Skalnik,D.G., Lievens,P.-J. and Orkin,S.H. (1992) Human CCAAT displacement protein is homologous to the *Drosophila* homeoprotein, Cut. *Nature Genet.*, **1**, 50–55.

Prowse,D.M., Bolgan,L., Molnar,A. and Dotto,G.P. (1997) Involvement of the Sp3 transcription factor in induction of p21Cip1/WAF1 in keratinocyte differentiation. *J. Biol. Chem.*, **272**, 1308–1314.

Russell,P. and Nurse,P. (1986) cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell*, **45**, 145–153.

Saha,P., Eichbaum,Q., Silberman,E.D., Mayer,B.J. and Dutta,A. (1997) p21CIP1 and Cdc25A: competition between an inhibitor and an activator of cyclin-dependent kinases. *Mol. Cell. Biol.*, **17**, 4338–4345.

Sheikh,M.S., Li,X.S., Chen,J.C., Shao,Z.M., Ordonez,J.V. and Fontana,J.A. (1994) Mechanisms of regulation of WAF1/Cip1 gene expression in human breast carcinoma: role of p53-dependent and independent signal transduction pathways. *Oncogene*, **9**, 3407–3415.

Sherr,C.J. and Roberts,J.M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163.

Shi,Y., Seto,E., Chang,L.S. and Shenk,T. (1991) Transcriptional repression by YY1, a human GLI-Kruppel-related protein and relief of repression by adenovirus E1A protein. *Cell*, **67**, 377–388.

Stein,G.S. and Borun,T.W. (1972) The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. I. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. *J. Cell Biol.*, **52**, 292–307.

Strausfeld,U., Labbe,J.C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Doree,M. (1991) Dephosphorylation and activation of a p34cdc2/cyclin B complex *in vitro* by human CDC25 protein. *Nature*, **351**, 242–245.

Thomas,P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl Acad. Sci. USA*, **77**, 5201–5205.

Tini,M., Fraser,R.A. and Giguere,V. (1995) Functional interactions between retinoic acid receptor-related orphan nuclear receptor (ROR alpha) and the retinoic acid receptors in the regulation of the gamma F-crystallin promoter. *J. Biol. Chem.*, **270**, 20156–20161.

Valarche,I., Tissier-Seta,J.P., Hirsch,M.R., Martinez,S., Goridis,C. and Brunet,J.F. (1993) The mouse homeodomain protein Phox2 regulates Ncam promoter activity in concert with Cux-CDP and is a putative determinant of neurotransmitter phenotype. *Development*, **119**, 881–896.

Vanwijnen,A.J. *et al*. (1996) CDP/Cut Is the DNA-binding subunit of histone gene transcription factor Hinf-D—a mechanism for gene regulation at the G (1)/S phase cell cycle transition point independent of transcription factor E2F. *Proc. Natl Acad. Sci. USA*, **93**, 11516–11521.

Waga,S., Hannon,G.J., Beach,D. and Stillman,B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369**, 574–578.

Whyte,P. (1995) The retinoblastoma protein and its relatives. *Semin. Cancer Biol.*, **6**, 83–90.

Xu,X. and Burke,S.P. (1996) Roles of active site residues and the NH_2-terminal domain in the catalysis and substrate binding of human Cdc25. *J. Biol. Chem.*, **271**, 5118–5124.

Yang,J.Q., Remmers,E.F. and Marcu,K.B. (1986) The first exon of the c-*myc* proto-oncogene contains a novel positive control element. *EMBO J.*, **5**, 3553–3562.

Yang,W.M., Inouye,C., Zeng,Y., Bearss,D. and Seto,E. (1996) Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc. Natl Acad. Sci. USA*, **93**, 12845–12850.

Yoon,S.O. and Chikaraishi,D.M. (1994) Isolation of two E-box binding factors that interact with the rat tyrosine hydroxylase enhancer. *J. Biol. Chem.*, **269**, 18453–18462.

Zeng,Y.X., Somasundaram,K. and el-Deiry,W.S. (1997) AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nature Genet.*, **15**, 78–82.

Zwicker,J., Gross,C., Lucibello,F.C., Truss,M., Ehlert,F., Engeland,K. and Muller,R. (1995a) Cell cycle regulation of cdc25C transcription is mediated by the periodic repression of the glutamine-rich activators NF-Yand Sp1. *Nucleic Acids Res.*, **23**, 3822–3830.

Zwicker,J., Lucibello,F.C., Wolfraim,L.A., Gross,C., Truss,M., Engeland,K. and Muller,R. (1995b) Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. *EMBO J.*, **14**, 4514–4522.

Zwicker,J., Liu,N.S., Engeland,K., Lucibello,F.C. and Muller,R. (1996) Cell cycle regulation of E2F site occupation *in vivo*. *Science*, **271**, 1595–1597.

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