

Functional cdc25C Dual-Specificity Phosphatase Is Required for S-Phase Entry in Human Cells

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In view of the common regulatory mechanism that induces transcription of the mitotic phosphatase cdc25C and cyclin A at the beginning of S-phase, we investigated whether cdc25C was required for S-phase transit. Here, we show that in both nontransformed human fibroblasts and HeLa cells, cdc25C protein levels significantly increased concomitant with S-phase onset and cyclin A synthesis. Activity measurements on immunoprecipitates from synchronized HeLa cells revealed a sharp rise in cdc25C-associated phosphatase activity that coincided with S-phase. Microinjection of various antisense-cdc25C molecules led to inhibition of DNA synthesis in both HeLa cells and human fibroblasts. Furthermore, transfection of small interfering RNA directed against cdc25C specifically depleted cdc25C in HeLa cells without affecting cdc25A or cdc25B levels. Cdc25C RNA interference was also accompanied by S-phase inhibition. In cells depleted of cdc25C by antisense or siRNA, normal cell cycle progression could be re-established through microinjection of wild-type cdc25C protein but not inactive C377S mutant protein. Taken together, these results show that cdc25C not only plays a role at the G2/M transition but also in the modulation of DNA replication where its function is distinct from that of cdc25A.

INTRODUCTION

Cyclin-dependent kinases (cdks) play a central role in the regulation of eukaryotic cell cycle progression (Morgan, 1995). Regulatory mechanisms responsible for their controlled activation include combinatorial assembly of cdks with one of a number of different cyclins, activating phosphorylation of a conserved threonine residue (T161 in human cdk1/cdc2), inhibition by association with specific inhibitors, and inhibitory phosphorylation of a conserved tyrosine (and adjacent threonine) in the amino-terminal ATP-binding region. Ultimately cdk/cyclin complexes are rapidly activated by dephosphorylation of this tyrosine (and

threonine), which is brought about by cdc25 dual specificity phosphatases (for review, see Nilsson and Hoffmann, 2000). Cdc25 phosphatases belong to the tyrosine phosphatase family. Their active site loop, containing the HCX5R motif, is similar to that of tyrosine phosphatases but their overall topology is different (Fauman *et al.*, 1998).

In human cells, cdc25 proteins are encoded by a multigene family, consisting of cdc25A, cdc25B, and cdc25C (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991). All three display more than 60% identity to each other within the carboxyl terminal, catalytic domain. The less-conserved amino terminal domains are multiply phosphorylated and harbor distinct regulatory and substrate specificity determinants (Hoffmann *et al.*, 1994; Strausfeld *et al.*, 1994; Gabrielli *et al.*, 1997). Amino terminal domains are also subject to alternative splicing giving rise to two, three, and five variants of cdc25A, B, and C, respectively (Baldin *et al.*, 1997; Bureik *et al.*, 2000; Wegener *et al.*, 2000). Cdc25A is required for G1/S transition (Jinno *et al.*, 1994) and seems to be regulated in part by mitogenic signaling (Galaktionov *et al.*, 1995).

More recent data suggest that in humans a stable form of cdc25A may also play a role in mitosis (Mailand *et al.*, 2002) and checkpoint signaling (Zhao *et al.*, 2002). Cdc25B is

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Abbreviations used: BrdU, 5 bromo-deoxyuridine; cdk, cyclin-dependent kinase; PMSF, phenylmethylsulphonyl fluoride; H1, histone 1.

present in S- and G2-phase cells. Overexpression of cdc25B in mammalian cells rapidly induces mitotic entry, overriding most cell cycle checkpoints (Karlsson *et al.*, 1999). Indeed cdc25B is required for the G2/M progression and a number of reports suggest that cdc25B is the initial activator of cdc2/cyclin B at mitotic entry (Gabrielli *et al.*, 1996; Nishijima *et al.*, 1997; Lammer *et al.*, 1998).

Likewise cdc25C has been associated with mitotic activation. There is strong evidence that cdc25C is involved in mitotic entry by dephosphorylating T14 and Y15 of cdc2/cyclin B. Its inhibition through microinjection of antibodies blocks entry into mitosis (Millar *et al.*, 1991; Seki *et al.*, 1992). Cdc25C becomes phosphorylated and activated at mitosis by cdc2/cyclin B itself and a positive feedback loop has been proposed (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994). Indeed, cdc25C phosphorylated by cdc2/cyclin B is capable of inducing partial mitotic activation (Hoffmann *et al.*, 1993; Strausfeld *et al.*, 1994). However, cdc25C is also subject to other site-specific phosphorylation events at mitosis, particularly by polo-like kinase 1 (Plk1; Toyoshima-Morimoto *et al.*, 2002) and undergoes pin1-dependent proline isomerization (Zhou *et al.*, 2000). Moreover, cdc25C is a converging point of several signal transduction cascades and links the mitotic activation of cdc2/cyclin B to the DNA replication and repair checkpoint (for review, see Walworth, 2001). Muller and colleagues have reported that cdc25C gene transcription is restricted to the S- and G2-phase, and the underlying transcriptional regulation is the same as found for cyclin A (Zwicker *et al.*, 1995). Cyclin A protein appears exclusively during S and G2 and is required for the initiation of DNA replication (Girard *et al.*, 1991). In light of these parallels to cyclin A expression we have addressed the question whether cdc25C had a role during S-phase and provide experimental evidence that in human fibroblast and HeLa cells, active cdc25C protein is required for proper G1-S progression even in the presence of cdc25A.

MATERIAL AND METHODS

Cell Culture, Synchronization, and Cell Cycle Analysis

Nontransformed human fibroblasts Hs68 (CRL-1365) and HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum. Hs68 fibroblasts were synchronized in G1 by serum deprivation and resynchronized for S and G2 using hydroxyurea (HU) as described elsewhere (Girard *et al.*, 1992). HeLa cells were synchronized by blocking the cells with thymidine at G1/S as described elsewhere (Stein *et al.*, 1998). Briefly, asynchronous HeLa cells were supplemented with 2 mM thymidine for 24 h. Cells were released from the thymidine block by incubation in PBS for 30 s and 5 min at 37°C followed by incubation in serum containing DME supplemented with 24 μ M deoxycytidine for 15 min. Finally, cells were refed with fresh media supplemented with 24 μ M deoxycytidine and grown for 12–14 h. At this point cells were refed with fresh media containing 2 mM thymidine for 24 h and subsequently released as described above. In some experiments mitotic cells were mechanically collected by mitotic shakes 10, 11, and 12 h after double thymidine block. Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS). Cells were trypsinized, and nuclei were stained for DNA in PBS, 0.1% Triton X-100, 5% glycerol, and 50 μ g/ml propidium iodide. Subsequently, fluorescence from propidium iodide-DNA complexes was determined using a FACScan flow cytometer and CellQuest analysis software (Becton Dickinson, Franklin Lakes, NJ).

Semiquantitative RT-PCR

Total RNA from synchronized or randomly growing Hs68 fibroblasts or HeLa cells was prepared using the RNeasy kit (Qiagen, Courtabouef, France). Total RNA, 0.25 μ g, was reverse-transcribed using a RNA PCR kit (Perkin Elmer-Cetus, Boston, MA). The resulting cDNA was then subjected to PCR amplification in 100- μ L reactions containing 10 mM Tris/Cl, pH 8.3, 1.5 mM MgCl, 50 mM KCl, 0.2 mM of each dATP, [α -³²P]dCTP (50 mCi/mmol), dGTP, and dTTP, 0.5 μ M forward oligonucleotide, GATGCCAGAGAACT-TGAAC (annealing to nucleotides 858–866 of cdc25C), 0.5 μ M reverse oligonucleotide, TGAAACCTAATCCATTCCC (annealing to nucleotides 1779–1797 of cdc25C), and 2.5 U of *Taq* polymerase. These oligonucleotides were designed to amplify a fragment in the 3' region of cdc25C, which is common to all described cdc25C splice variants (Bureik *et al.*, 2000; Wegener *et al.*, 2000). Twenty-five amplification cycles with the following temperature profile were performed: denaturation at 95°C for 15 s, primer annealing at 55°C for 30 s, and primer elongation at 72°C for 2 min. These conditions allowed detection of cdc25C transcripts during all cell cycle stages and were in the linear range of amplification (our unpublished results). Reactions were analyzed on ethidium-bromide-containing 1.4% agarose gels or on 5% polyacrylamide gels followed by autoradiography.

Antibodies and Immunoblot Analyses

Antiserum was raised against the peptide MSTELFSSTREEGSSGS-GPS corresponding to the N-terminus of cdc25C. Immunizations and subsequent affinity purification of the antibody N20 were performed as previously described (Turowski *et al.*, 1999). This antibody was highly specific for in vitro-translated or recombinant cdc25C as well as the endogenous protein when used in immunoprecipitations or immunoblots (see also Figure 2F).

Total extracts of HeLa or Hs68 cells were electrophoretically separated on 10% SDS-polyacrylamide gels and immunoblotted as described (Turowski *et al.*, 1999). Primary antibodies against cdc25A (sc7389, Santa Cruz Biotechnology, Santa Cruz, CA), cdc25B (sc326, Santa Cruz Biotechnology, and clone 23, Transduction Laboratories, Becton Dickinson, San Diego, CA), cdc25C (C20: sc327, Santa Cruz Laboratories, and N20), anti-cdk2 (M2, sc163, Santa Cruz Laboratories), and cyclin A (Girard *et al.*, 1991) were used at 1:500. Anti-tubulin ascites (clone DM1A; Blose *et al.*, 1984) was used at 1:10,000.

Immunoprecipitation and Phosphatase Activity Measurement

Active cdc25C was immunoprecipitated from double thymidine synchronized HeLa cells using anti-cdc25C-C20 or N20 antibodies. Briefly, 5 μ g anti-cdc25C were incubated with 20 μ L protein G-sepharose (AP-Biotech, Amersham, UK) for 2 h at 4°C. Synchronized HeLa cells from a 100-mm culture dish were washed twice in ice-cold PBS, scraped into PBS, and collected by centrifugation at 1000 \times g for 5 min. Cells were lysed in RIPA buffer (50 mM Tris/Cl, pH 8.0, 50 mM NaCl, 1.0 mM EDTA, 0.5% sodium deoxycholate, 1.0% Triton X-100, 50 nM calyculin A, 2 μ g/ml aprotinin, 4 μ M leupeptin, 1 mM PMSF, 3 μ M pepstatin), and the soluble lysate was precleared with protein G-Sepharose for 30 min at 4°C. Subsequently 20 μ L of antibody-protein G-Sepharose complex was added to lysate containing 500 μ g of protein. After incubation for 2 h, antibody-protein G complexes were collected by centrifugation and washed twice with RIPA buffer, twice with RIPA containing 400 mM NaCl, twice in TBS (50 mM Tris/Cl, pH 7.5, 150 mM NaCl), and twice in TBS containing 1.0 mM EDTA before activity measurements were made.

Cdc25C activity was measured using 3-O-methyl fluorescein phosphate (3-OMFP) essentially as described by Gottlin *et al.* (1996). 3-OMFP was resuspended at a final concentration of 150 μ M in 50 mM Tris/Cl, pH 8.0, 50 mM NaCl, 5 mM DTT, 0.1% (wt/vol) BSA, 1.0 mM EDTA. Pellets of immunoprecipitated cdc25C beads were

resuspended in 300 μ L of 3-OMFP assay buffer with and without 2 mM sodium orthovanadate. After incubation at 30°C for 30 min fluorescence was measured on a Wallac Victor2 fluorescence plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA) using 489-nm excitation and recording at 531 nm. Experiments were done in duplicates and repeated at least three times. Fluorescent emissions values were corrected for background activity, which was immunoprecipitated from HeLa cells double blocked in thymidine and which generally gave the same values as immunoprecipitates without antibody.

Microinjection and Immunofluorescence

Cdc25C antisense oligonucleotides AS1 (TGAGAAGAGTTCGTA-GACAT), AS2 (CCTTGAATTTTCCACCTGCT), the corresponding sense oligonucleotides S1 and S2, and cdc25B antisense oligonucleotide (CCGGCCCCGCGGATGGAGGTGC) were synthesized using phosphorothioate modification of end bases to increase in vivo half-life and purified by reversed-phase chromatography (Eurogentec, Seraing, Belgium). For the sense and antisense cdc25C expression vectors p25Cs and p25Cas, the open reading frame (nucleotides 211-1632) from human cdc25C cDNA (Sadhu *et al.*, 1990) was subcloned into pJ3 (Morgenstern and Land, 1990).

Synchronized HS68 or HeLa cells were microinjected as described previously (Lamb and Fernandez, 1997). Before injection, plasmids, oligonucleotides, and proteins were diluted into injection buffer containing 0.5 mg/ml inert marker antibodies (to act as a marker for injected cells). Plasmids were injected at <100 copies per cell (ca. 10 nM in the needle) and oligonucleotides at concentrations of <5 nM. Higher concentrations tended to affect S-phase nonspecifically. Cdc25C-wt and C377S proteins were at a concentration of ca. 0.2 mg/ml after dilution into marker-containing injection buffer. Cells were microinjected at indicated times throughout G1 or early S-phase and further subcultured in presence of bromodeoxyuridine (BrdU). At various time points after microinjection, cells were fixed and stained for incorporated BrdU, DNA, and microinjection marker (Girard *et al.*, 1991). Cells were mounted and photographed as described elsewhere (Turowski *et al.*, 1999).

Recombinant cdc25C Protein

Human Cdc25C subcloned into the T7-inducible bacterial expression vector pRK171 was a kind gift of Paul Russell (Scripps Research Institute, La Jolla, CA). Recombinant cdc25C was expressed using BL21 (DE3) bacteria transformed with the pRK171-hcdc25C construct and cdc25C-containing inclusion bodies isolated as previously described (Strausfeld *et al.*, 1991). Instead of the renaturation procedure described before, inclusion bodies were solubilized in guanidinium-containing buffer, and proteins were renatured by quick dilution. After overnight dialysis cdc25C was further separated from contaminants and improperly folded protein by anion exchange chromatography on a Mono Q column. Details of this procedure will be published elsewhere and are available on request. The plasmid pRK171-hcdc25C was mutated using the Quickchange Mutagenesis kit (Stratagene, La Jolla, CA) and mutant oligonucleotides (top strand: ATAATCATCGTGTCCACAGTGAGT-TCTCCTCAGAGAGGG and the corresponding lower strand oligonucleotide). The mutated construct was verified by sequencing and encoded human cdc25C with a single cysteine to serine change at amino acid position 377 (cdc25C-C377S). Subsequently, the C377S protein was expressed and purified in the same way as wild-type protein. Specific phosphatase activities were measured using para-nitrophenylphosphate (pNPP) as a substrate (Gottlin *et al.*, 1996). Wild-type cdc25C had a specific activity of 46 nmol/min/mg, whereas the C377S mutant protein displayed no activity toward pNPP at all.

RNA Interference

The small interference RNA (siRNA) duplexes were 21 base pairs. They were selected from within the open reading frames of cdc25A,

B, and C ~70 base pairs downstream of the ATG at the next double adenine. The sequences of the coding strands of the human cdc25A, B, and C siRNA duplexes were AAGGCGCUAUUUGGCGCUUCA, AAGAGCGAGGCGGGCAGUGGA, and AAAAAUGUUGCCUC-GAUCUUUC, respectively. The control siRNA used was ACCGCGCCGAGGUGAAGUU, targeting enhanced green fluorescent protein (Cortez *et al.*, 2001). siRNA were synthesized and annealed by Dharmacon Research Inc, (Lafayette, CO). For RNA interference siRNA duplexes were resuspended in RNase-free water at 20 μ M and transfected as described elsewhere (Liu and Erikson, 2002). Alternatively, cells were transfected using calcium phosphate (Alessi *et al.*, 1996). Cells were incubated in the presence of the antisense RNA for 24 h. Cells were washed and further cultured in the absence or presence of BrdU for 12–24 h before they were processed for immunofluorescence, microinjection, or Western blotting. To determine transfection efficiency, cells were transfected with siRNA directed against GFP coupled to fluorescein. After 24-h transfection, cells were washed once in PBS, and the proportion of cells positive for fluorescein was determined.

RESULTS

Cell Cycle Analysis of cdc25C Expression and Activity

Human fibroblasts Hs68 were synchronized by serum starvation and RNA was isolated at various times after serum stimulation during the cell division cycle. RT-PCR analysis revealed an ca. 5–10-fold increase of cdc25C message levels during S- and G2-phase (Figure 1A). This observation is in line with previous reports showing that human cdc2, cyclin A, and cdc25C genes are subject to S/G2-specific transcription (Lucibello *et al.*, 1995; Zwicker *et al.*, 1995). For cdc2, transcriptional changes are without consequence on steady state protein levels (McGowan *et al.*, 1990), whereas for cyclin A, protein and transcript levels are tightly linked (Pines and Hunter, 1990). We analyzed whether S/G2-specific transcription of human cdc25C was also linked to G1/S-phase specific protein induction (Figure 1B). Low amounts of cdc25C were detected in quiescent cells and throughout the majority of G1. At the G1/S transition, coinciding with the appearance of cyclin A (Girard *et al.*, 1991), cdc25C protein levels markedly increased. The amount of cdc25C further increased throughout S and G2, reaching maximal levels in late G2 and mitosis. To discount that this induction of cdc25C was due only to growth factor readdition after serum starvation rather than regulated by the cell cycle, HeLa cells were synchronized mechanically (by mitotic shake), and cdc25C levels were analyzed at various times after replating (Figure 1, C and D). Cdc25C message levels were high as cells entered G1 and reached a maximum 4 h after the mitotic shake. Thereafter message levels dropped markedly to attain a relative minimum in late G1, just before S-phase. During S and G2 message levels again increased strongly similarly to those observed in Hs68 cells (Figure 1C). Cdc25C protein levels paralleled these message levels (Figure 1D). They were high in mitosis and decreased throughout the following G1 phase to reach a minimum in late G1. At S-phase entry, concomitant with cyclin A synthesis, we again observed a major increase in cdc25C. The G1 decrease in cdc25C protein levels observed in HeLa cells was found in four independent experiments with cells synchronized by either mitotic shake or nocodazole. Taken together from these data, it appeared that G1 fibroblasts entering the cell cycle from a quiescent state hardly contain any

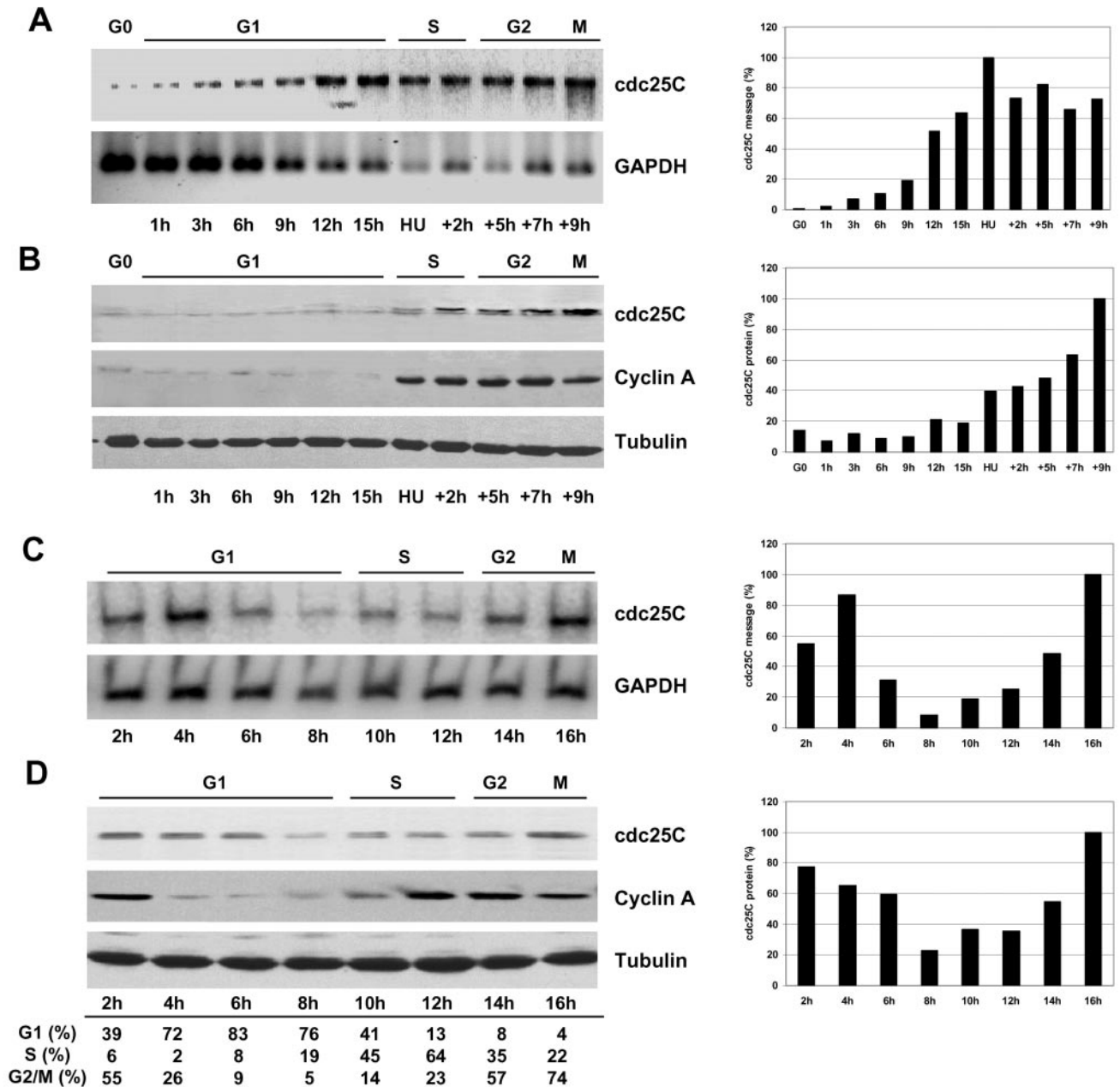


Figure 1. S/G2-specific expression of *cdc25C* in human cell lines. (A and B) Hs68 fibroblasts were synchronized by serum starvation for G1 and resynchronized for S and G2 using hydroxyurea (HU). Under these conditions, HS68 cells were in G1 from 0 to 16 h after refeeding and in case of resynchronization using HU, cells were in S during the first 4 h after release from HU block, and in G2 in the following 5 hours, with a peak of mitosis (M) at around 9 h after HU release (Girard *et al.*, 1992). (A) Total RNA from HS68 fibroblasts was isolated at the indicated time points, and 0.25 μ g was subjected to RT-PCR using specific primers for *cdc25C* or GAPDH as detailed in MATERIALS AND METHODS. Subsequently, levels of *cdc25C* and GAPDH messages were revealed by PAGE and autoradiography. Radioactivity of *cdc25C* RT-PCR products was recorded, normalized to the corresponding GAPDH values, and expressed as percent of maximal measured value (histogram on the right). (B) Cells were lysed at the indicated time points, and ~ 40 μ g of total cellular proteins was separated electrophoretically and immunoblotted for *cdc25C* (top panel), cyclin A to indicate S-phase onset (middle), and tubulin as loading control (bottom). Densitometric quantification of *cdc25C* protein levels, normalized to the corresponding tubulin levels, is shown on the right (expressed as percentage of maximal measured value). (C and D) Mitotic HeLa cells were collected by mitotic shake and recultured. At the indicated times RNA (C) and protein (D) levels were analyzed as detailed for A and B. The cell cycle phases indicated below the mitotic release times in D, were determined by FACS for the same cell sample as analyzed.

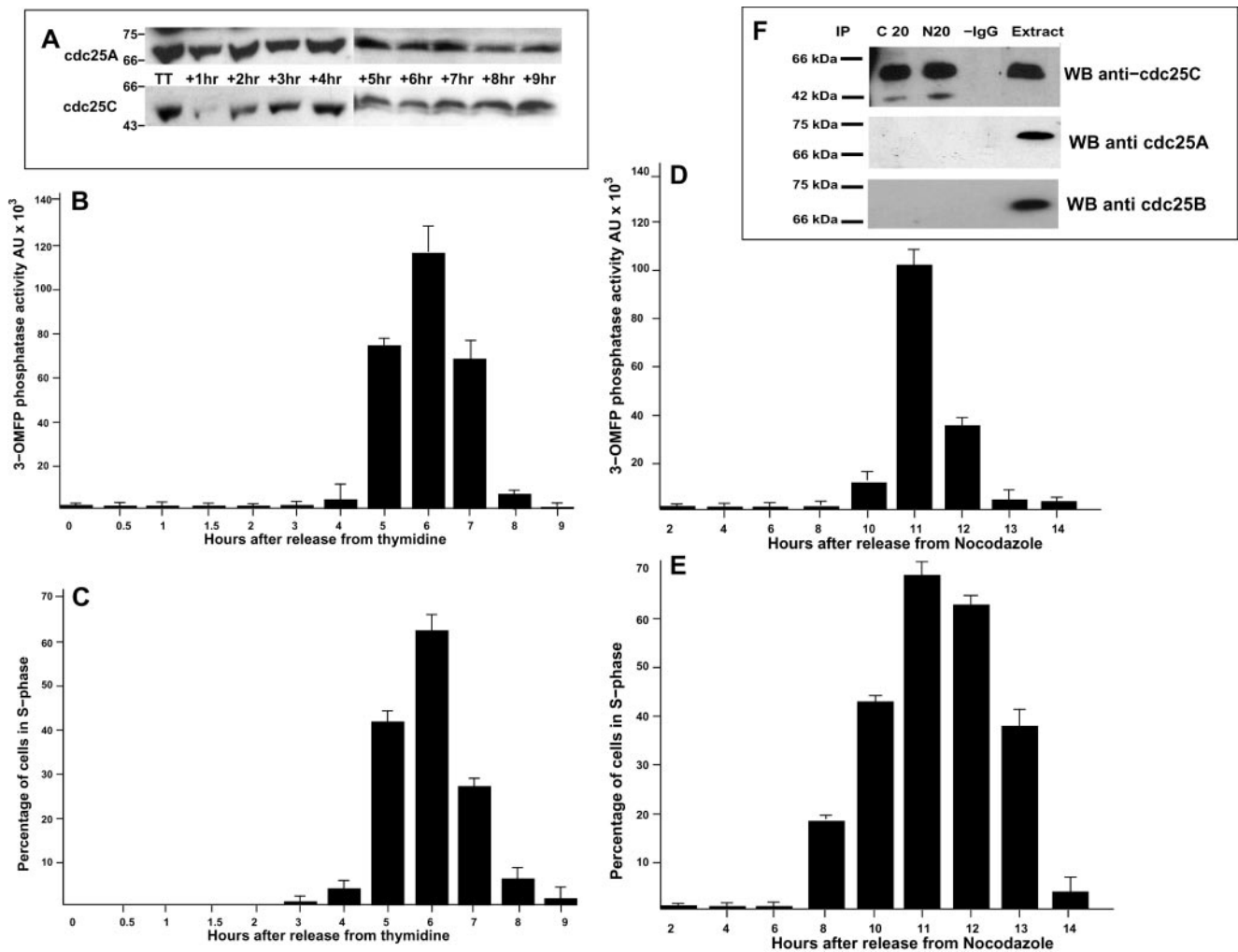


Figure 2. *cdc25C* activity transiently increases during S-phase. (A). HeLa cells were synchronized before S-phase by double thymidine block. At different times after washing out thymidine, cells were lysed and analyzed by Western blot for the expression of *cdc25A* (top panel) and *cdc25C*. Protein extracts were quantitated using Bradford assay (B), and equal amounts of cell extract were loaded for each lane. Western blots show typical examples obtained in four different experiments. Alternatively, HeLa cells were synchronized by double thymidine block or nocodazole and at different times after release were lysed for *cdc25C* activity measurements or fixed for analysis of BrdU incorporation. *cdc25C* activity was assessed by immunoprecipitation under nondenaturing conditions. Release of phosphate from 3-OMFP was measured by the associated increase in fluorescence. 3-OMFP phosphatase activity of *cdc25C* immunoprecipitates isolated at different times after release from double thymidine block. The time points correspond to those analyzed by Western blot in A. (C) The incorporation of BrdU in cells at the same time points. (D) A similar analysis of *cdc25C* activity in cells released from a nocodazole block and the corresponding data for BrdU incorporation (E). Inset in F: a Western blot analysis of the immunoprecipitate corresponding to the 6-h time point in A and B. Immunoprecipitates made with either C20 or N20 anti-*cdc25C* antibodies were blotted for the presence of *cdc25C* (top), *cdc25A* (middle) and *cdc25B* (bottom). Histograms are the mean values obtained in three different experiments. Activity is expressed in arbitrary fluorescence units compared against the levels in double thymidine blocked cells, which are essentially the same as those detected in an assay without immunoprecipitate.

cdc25C, and notable de novo synthesis of RNA and *cdc25C* protein occurred at the G1/S-phase boundary. Likewise in cycling HeLa cells, we observed a clear decrease of *cdc25C* during G1 and induced expression at the G1/S boundary.

As shown in Figure 2A, thymidine-blocked HeLa cells (TT) contained steady state levels of *cdc25C* similar to those observed in G1 cells. However, on release from the thymidine block, *cdc25C* levels rapidly decreased for 60 min before de novo synthesis occurred, followed by a consistent

increase in *cdc25C* levels over the next 8 h. In contrast, levels of *cdc25A* remained constant during the same period. We next asked if these changes in *cdc25C* expression were also reflected by changes in *cdc25C* activity. Considering the low abundance of *cdc25C*, we chose to immunoprecipitate the protein from synchronized human fibroblasts or HeLa cells and to measure activity using the highly sensitive fluorescent substrate 3-OMFP (Gottlin *et al.* 1996). As shown in Figure 2B, as HeLa cells were released from a thymidine

block, no cdc25C activity was observed during the first 4 h. However, cdc25C activity increased sharply 5–6 h after thymidine release before falling again. This peak in cdc25C activity coincided with maximal S-phase activity as determined by BrdU incorporation (Figure 2C). A similar result was obtained in HeLa cells released from a nocodazole block (Figure 2D). Again the peak of cdc25C activity coincided with the peak of S-phase activity (Figure 2E). Cdc25C phosphatase activity at S-phase constituted only a fraction of that measurable at mitosis. Typically, cdc25C immunoprecipitated from M-phase extracts (blocked and released from nocodazole) and measured under the same conditions as for S-phase showed 3–5-fold higher activity than peak S-phase samples (our unpublished results). However, this increase in activity at S-phase reverted to background levels as cells entered G2. As shown in Figure 2F, the phosphatase activity measured was specific to cdc25C because our cdc25C antibodies quantitatively immunoprecipitated cdc25C but not even traces of cdc25A or B. A similar increase in cdc25C activity was also detected in synchronized HS68 cells, with the peak of cdc25C activity detectable 18.5 h after refeeding (corresponding to mid-S-phase, our unpublished results). These data show that cdc25C is specifically synthesized at S-phase entry in refed cells and in cycling cells. In addition, this *de novo* synthesis of cdc25C is accompanied by a peak in cdc25C activity, which also coincides with maximal DNA synthesis. Interestingly, our observation that this activity was restricted to S-phase decreasing as cells transit into G2, strongly suggests regulation by posttranslational modification because the levels of cdc25C protein continue to increase, whereas the S-phase activity declines (see also DISCUSSION).

Antisense Depletion of cdc25C in Human Cells

With low levels in G1, induction during S and maximal levels during G2/M, the observed cdc25C expression pattern is reminiscent of that seen with cyclin A (Pines and Hunter, 1990). Cyclin A has been shown to be required for S-phase initiation (Girard *et al.*, 1991), whereas no such role has so far been described for cdc25C. To examine if the induction of cdc25C protein expression and activity was associated with a potential role of cdc25C at S-phase, we chose to specifically interfere with its protein resynthesis using different antisense approaches (Figure 3A). On the one hand we used the expression constructs p25Cs and p25Cas, containing the full-length open reading frame of human cdc25C under the control of an SV40 promoter in sense or antisense orientation, respectively. Alternatively, because mammalian cdc25s share strong sequence identity within their catalytic domain, antisense oligonucleotides targeting the most specific and divergent regions of cdc25C were designed. The sequences of single-stranded DNA oligonucleotides AS1 and AS2 were unique to cdc25C and absent from cdc25A or B. To act as controls, oligonucleotides encoding the sense strands were used.

To analyze the effect of cdc25C antisense tools on S-phase transition, HS68 fibroblasts were synchronized by serum deprivation and microinjected at different times after entry into G1. Cells were fixed and analyzed 20–22 h later when most cells have completed DNA replication (Girard *et al.*, 1992). Transition through S-phase was assessed by monitor-

ing BrdU incorporation. Microinjection of oligonucleotides AS1 or AS2 effectively prevented BrdU incorporation, whereas microinjection of either control oligonucleotides S1 or S2 did not affect S-phase transition (Figure 3, B, C, and F). Notably, microinjection of oligonucleotide AS1 still blocked S-phase entry when injected as late as 15 h after serum stimulation (Table 1), which corresponded to the time in late G1 just before the marked increase in cdc25C protein (Figure 1B). Similar S-phase inhibition was observed in cells microinjected with the antisense-cdc25C expressing construct p25Cas (Figure 3F). Microinjection of either the sense construct p25Cs or marker antibodies had no effect on DNA synthesis. When microinjected into synchronized HeLa cells p25Cas, AS1 or AS2 efficiently blocked S-phase as well (Figure 3, D and E). This inhibition of S-phase was specific to antisense cdc25C and was not observed using antisense cdc25B. Neither microinjection of an antisense-cdc25B RNA expressing construct nor cdc25B antisense oligonucleotides prevented S-phase transition in Hs68 or HeLa cells (Figure 3F and our unpublished results).

siRNA to cdc25C Specifically Suppresses cdc25C Expression

To further confirm the specific requirement for cdc25C expression for S-phase, we exploited the recently described siRNA technique (Elbashir *et al.*, 2001). To specifically target cdc25C expression and deplete cdc25C protein in HeLa cells, we designed siRNA with a sequence located within the open reading frame of human cdc25C. Control siRNA duplexes targeting cdc25A, cdc25B, cyclin A, or enhanced green fluorescence protein (EGFP; Cortez *et al.*, 2001) were also used. Asynchronously growing HeLa cells were transfected with siRNA targeting human cdc25 proteins or EGFP and after 36–48 h were harvested for Western blotting. Transfection of control siRNA targeting a sequence in EGFP did not affect protein levels of cdc25A, B, or C (Figure 4). Transfection of siRNA targeting cdc25A, B, or C caused a specific and complete downregulation of each protein targeted (Figure 4). Strikingly, the transfection of siRNA targeting each of the three cdc25 isoforms did not affect the steady state protein levels of the other two. As shown, in cells transfected with siRNA to cdc25C, the levels of cdc25A (A) or cdc25B (B) were the same as those in nontransfected cells (NT) or cells transfected with siRNA to GFP. Densitometric analyses of many such immunoblots revealed that the changes in cdc25A or B were 5% or less in cells transfected with siRNA targeting cdc25C. That the level of cdc25A or cdc25B protein remained unchanged with respect to nontransfected cells after depletion of cdc25C is noteworthy because it implies that cells did not compensate for the lack of cdc25C by upregulating cdc25A or B. Similar results were obtained when HeLa cells were synchronized before the siRNA transfection. Cdc25C levels could be effectively knocked out within 12 h after release from a double thymidine block when using cdc25C siRNA, again suggesting that *de novo* synthesis of cdc25C occurs at S-phase. From these data we concluded that any result obtained with cdc25C siRNA was specifically due to the suppression of cdc25C protein expression and not to unspecific effects on cdc25A or B. Indeed when cells were transfected with siRNA to cdc25A and B, we again observed a similar isoform specific knockdown of either cdc25A (Figure 4C) or cdc25B (Figure 4D). In none of

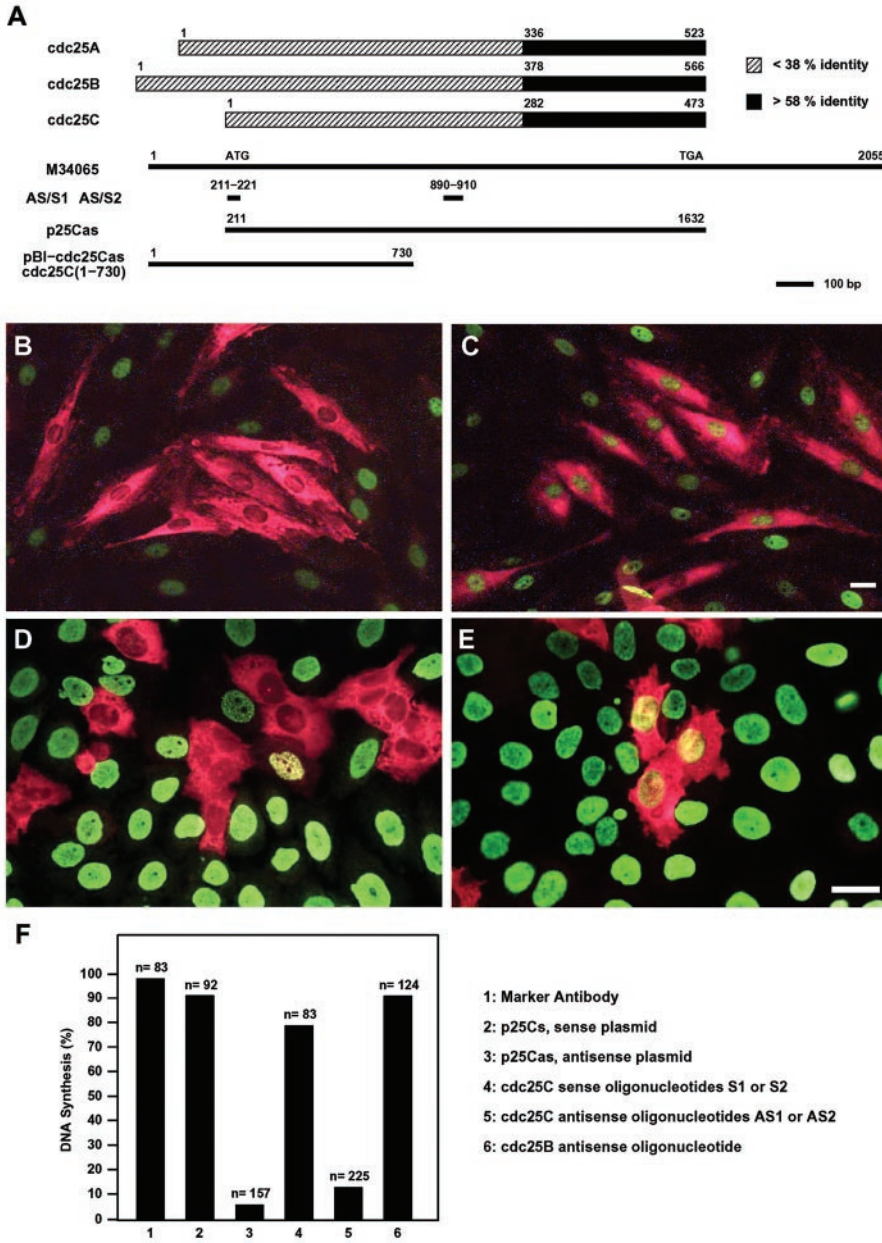


Figure 3. Microinjection of antisense-cdc25C prevents S-phase entry. (A) cdc25A, B, and C protein sequences are schematically aligned to each other. Numbers correspond to the amino acids of the published sequences (GenBank accession numbers M81933, M81934, and M34065, respectively). The catalytic domains (black) display more than 58% identity, the amino terminal domains (white) <38% identity. The cDNA of cdc25C (Sadhu *et al.*, 1990; M34065) is depicted underneath to illustrate the position of the antisense and sense (control) oligonucleotides AS/S1 and AS/S2 and the fragments used for the antisense vectors p25Cas. The positions in base pairs are given relative to M34065. (B and C) Hs68 fibroblasts were synchronized by serum starvation and refed with medium containing serum and BrdU. Ten hours after serum readdition, cells were microinjected with cdc25C antisense AS1 (B) or sense S1 (C) oligonucleotide, together with inert rabbit IgG. Cells were fixed 22 h after refeeding (when >95% of cells have initiated or completed DNA synthesis) and double-stained for the rabbit marker antibodies (red) and BrdU incorporation (green). (D and E) Mitotic HeLa cells were obtained using thymidine followed by nocodazole treatment. Three hours after relieving the mitotic block, cells were microinjected with oligonucleotides AS1 (D) or S1 (E) and marker antibody. BrdU was added and the cells were cultured for another 12 h before fixation. Shown are merged micrographs of cells double-stained for marker antibody (red) and BrdU (green). Scale bars, 10 μ m. The histogram in F summarizes the data obtained from four to six independent sets of microinjection experiments performed with synchronized Hs68 fibroblasts. The experimental outline was as described in B and C, except that p25Cs (2) and p25Cas (3) were injected in the first 5 h after the refeed to allow efficient expression. In all cases cells were fixed 22–24 h after the refeed. Values in columns 4 (and 5) represent data from experiments performed with either oligonucleotides S1 or S2 (or AS1 or AS2). Percent of DNA synthesis refers to the percentage of microinjected cells that have incorporated BrdU. n = number of injected cells.

the cases where a cdc25 isoform was targeted did we see changes in the levels of the other cdc25 isoforms when compared with GFP siRNA-transfected or nontransfected cells (Figure 4, A–D).

Cyclin A and cdk2 were also examined after cdc25C siRNA transfection: Cyclin A was strongly reduced in cells transfected with siRNA to cyclin A as expected, but unchanged in cells transfected with cdc25C (Figure 4E). We also examined the phosphorylation state of cdk2 as a potential substrate for cdc25C at S-phase. HeLa cells were depleted of cdc25C by siRNA, and cdk2 was examined by Western blot using long gels run at pH 8.6. This has previously been shown to allow immunodetection of two bands

for cdk2: the top band being the dephosphorylated active form and the lower band corresponding to the phosphorylated, inactive form (Dulic *et al.*, 1993). As shown in Figure 4F, mainly the 33-kDa top band was detectable in control cells transfected with siGFP. When cells are depleted of cdc25C, the majority of cdk2 still resolves as the slow-migrating, active form, but a small proportion now clearly resolves in the fast migrating form. Membranes were re-probed for cdc25C to ensure that effective knockdown of cdc25C had occurred (our unpublished results). These results suggest that under these conditions, cdk2 was not a direct target for cdc25C. In support of this we did not find significant changes in cdk activity in cyclin A or cdk2 im-

Table 1. Inhibition of DNA synthesis by antisense-cdc25C oligonucleotides in mid- and late-G1

| Microinjection | No. of cells injected | Number of cell positive for BrdU | % cells inhibited |
|--|-----------------------|----------------------------------|-------------------|
| Sense cdc25C oligonucleotide S1 after 10 h | 106 | 98 | 8 |
| Sense cdc25C oligonucleotide S1 after 15 h | 36 | 34 | 6 |
| Antisense cdc25C oligonucleotide S1 after 10 h | 168 | 17 | 90 |
| Antisense cdc25C oligonucleotide S1 after 15 h | 103 | 31 | 70 |

Background DNA synthesis was 97%. Hs68 fibroblasts were synchronized by serum starvation. In mid- and late-G1, at 10 or 15 h after the refeed, cells were microinjected with marker antibodies and sense (S1) or antisense oligonucleotide (AS1). After injection cells were further cultured in the presence of BrdU and fixed 22 h after refeeding. Subsequently, cells were stained for microinjection marker (to give the number of cells injected) and for DNA synthesis using anti-BrdU (to give the number of cells that have entered S-phase). None of the oligonucleotides inhibited DNA synthesis significantly when microinjected into S-phase cells, i.e., 18 to 22 h after the refeed. Numbers represent mean values of two to four independent experiments.

munoprecipitates isolated from cdc25C depleted cells (our unpublished results).

siRNA to cdc25C Inhibits DNA Synthesis

We next examined the consequences of cdc25C siRNA on DNA synthesis. As shown in Figure 5, HeLa cells transfected with cdc25C siRNA failed to incorporate BrdU during the following 24-h period. Little or no BrdU staining was seen in cells transfected with siRNA to cdc25C (Figure 5, C and D). The only cells positive for BrdU were in mitosis. In comparison, control cells transfected with EGFP siRNA showed BrdU staining in ~40% of cells (Figure 5, A and B). In all experiments the levels of transfection were very high (ca. 95%), as assessed using a fluorescently tagged siRNA targeting GFP. Transfections for shorter periods of time before BrdU addition resulted in a higher background levels of BrdU incorporation, most likely reflecting the half-life of the cdc25C protein present at the time of transfection. A similar failure to undergo DNA synthesis was also observed in cells transfected with cdc25A (Figure 5, E and F) but not cdc25B siRNA (our unpublished results), consistent with their previously reported functions at G1/S and mitosis, respectively (Jinno *et al.*, 1994; Gabrielli *et al.*, 1996).

Cdc25C Protein Injection into cdc25C-depleted Cells Restores S-Phase

Although the described cdc25C-depletion experiment seemed to specifically target cdc25C with respect to the other cdc25 isoforms, there remained the possibility that they interfered with another as yet unidentified cellular process. To further specify the role of cdc25C at S-phase entry, functional rescue experiments were performed. Human wild-type (wt) cdc25C was expressed in *Escherichia coli* and purified to apparent homogeneity (Figure 6A, lane 1). For catalysis all dual-specificity protein phosphatases depend on an essential cysteine residue in their active site (see Fauman *et al.*, 1998 and references therein). We mutated the catalytic cysteine in human cdc25C (C377) to serine to produce cdc25C-C377S. The C377S protein was expressed and purified in a way similar to that of the wt protein (Figure 6A, lane 2). Phosphatase activity of both proteins was assessed using inactive cdc2/cyclin B complexes immunoprecipitated from S/G2 HeLa cells.

As shown in Figure 6B, wild-type cdc25C but not C377S mutant protein strongly activated the histone H1 kinase activity of cdc2/cyclin B in a vanadate-sensitive manner, showing the functional integrity of these proteins. Hs68 fibroblasts were microinjected with p25Cas together with Lucifer Yellow to act as an injection marker. Twenty-two hours after injection when cells were blocked before S-phase, fluorescent cells were reinjected with wt or C377S cdc25C, and subsequent DNA synthesis was monitored (Figure 6, D–G). Active, wild-type cdc25C specifically restored the ability to enter S-phase (Figure 6, D and E; Table 2). In contrast, inactive cdc25C-C377S could not relieve the S-phase block in antisense cdc25C-depleted cells (Figure 6, F and G; Table 2), suggesting that cells need active cdc25C phosphatase to enter S-phase. Similar results were also obtained in cell microinjected with antisense DNA oligonucleotides (Table 2).

Functional rescue experiments were also performed in cells depleted of cdc25C through RNA interference. As shown in Figure 7 and in confirmation of the results shown in Figure 6, microinjection of active cdc25C into HeLa cells effectively overcame the cell-cycle block induced by cdc25C siRNA because injected cells underwent DNA synthesis within 4 h (Figure 7, A, C, and E; Table 3). Again the microinjection of inactive cdc25C-C377S mutant protein was unable to restore DNA synthesis (Figure 7, B, D, and F; Table 3).

Taken together, these data show that the failure to progress through S-phase in human nontransformed cells after ablation cdc25C results from the specific lack of cdc25C activity. This activity is required despite the presence of unaltered levels of cdc25A, suggesting a function distinct from that of cdc25A.

DISCUSSION

Cdc25C mRNA was originally described to be expressed primarily in the G2-phase of the cell cycle (Sadhu *et al.*, 1990; Jinno *et al.*, 1994). Studies based on more sensitive PCR technology showed that cdc25C transcripts appear from late G1 onward and that high levels are already reached in S-phase (Lucibello *et al.*, 1995). We observed a similar expression pattern in synchronized human fibroblasts Hs68 and HeLa cells. This stringently controlled, cell cycle-dependent transcription, which is also found for the cdc2 and cyclin A genes, is the result of repression by a cell-cycle-

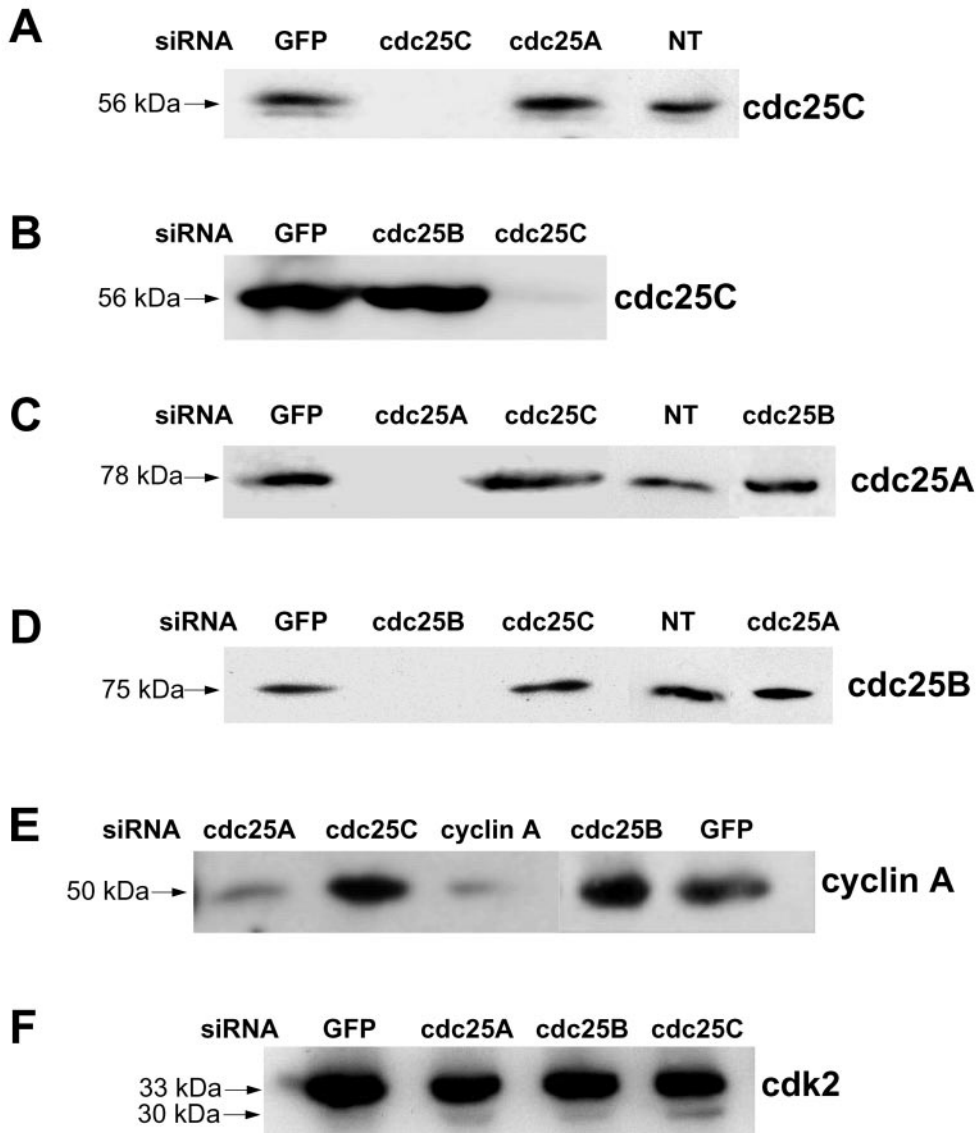


Figure 4. si-RNA specifically deplete cells of cdc25A, B, or C. Asynchronous HeLa cells were grown to ca. 50% confluence. They were then transfected with siRNA duplexes targeting EGFP (acting as a control, lanes GFP), cdc25A, cdc25B, or cdc25C. Thirty-six hours thereafter cells were harvested and total protein extracts were prepared. Samples were quantified by Bradford protein assay, and ~20 μ g of total cellular proteins was electrophoretically separated and transferred onto nitrocellulose. Equal loading between different lanes on the same blots was verified by Ponceau Red-coloration of the membranes. Subsequently, membranes were immunodecorated for cdc25C (A and B), cdc25A (C), cdc25B (D), cyclin A (E), or cdk2 (F). NT, nontransfected cells showing the endogenous levels of cdc25 proteins in these cells. Arrows in F are the positions of the slower-migrating active form or fast-migrating inactive form of cdk2.

dependent element during G1 (Zwicker *et al.*, 1995). Cdc25C protein levels were reported to fluctuate very little during cell cycle progression, but a certain increase has always been observed at S-phase (Millar *et al.*, 1991; Patel *et al.*, 1999).

We report here that in highly synchronized Hs68 fibroblasts cdc25C protein levels were markedly induced at the G1/S transition, subsequent to the rise in mRNA levels and parallel to cyclin A accumulation. Moreover, we found a similar induction at the G1/S transition of postmitotically synchronized HeLa cells or HeLa cells released from a thymidine block. Changes in cdc25C protein levels were not as marked as those found for cyclin A, especially in cycling HeLa cells (Figure 1D). Also, de novo cdc25C protein synthesis in refed Hs68 occurred significantly later than the induction of the RNA (Figure 1, A and B). This raises the possibility that cdc25C unlike cyclin A is additionally regulated on the translational level. However, in contrast to the

rapid protein degradation typically seen for cyclins (Morgan, 1995), the decrease of cdc25C after mitosis was slow.

We present experimental evidence that cdc25C displays significant phosphatase activity coinciding with DNA synthesis. Activity started to be detected at cell cycle times when protein levels also markedly increased. However, activity induction was much stronger than the increase in protein. Moreover cdc25C phosphatase activity was restricted to S-phase despite protein levels continually rising throughout G2. Therefore and in analogy to mitotic activation of cdc25C, posttranslational modification appears to regulate its S-phase-specific activity as well. It will be interesting to determine whether cdc25C activity at S-phase is modulated by direct posttranslational modifications such as phosphorylation as is the case in mitosis (Strausfeld *et al.*, 1994), changes in conformation such as isomerization (Zhou *et al.*, 2000), or binding to 14.3.3 (Peng *et al.*, 1997). We used

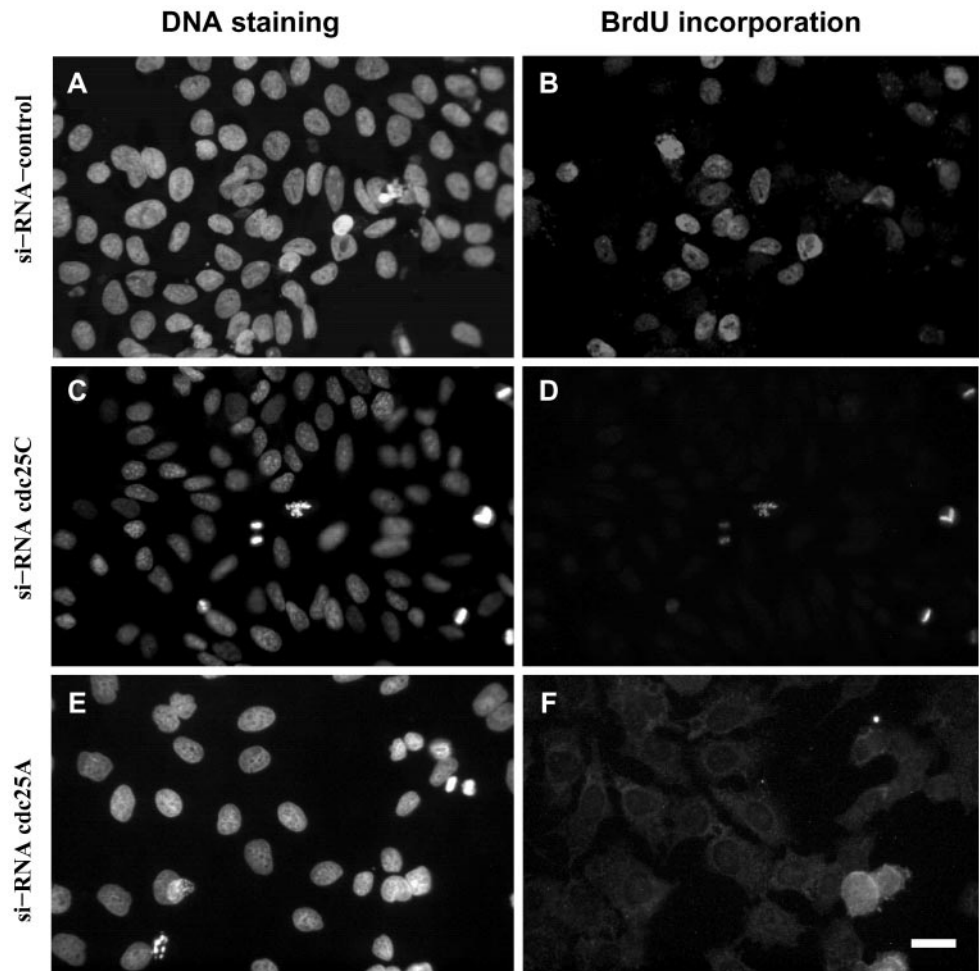


Figure 5. SiRNA duplexes targeting *cdc25A* and *cdc25C* block DNA synthesis in HeLa cells. Asynchronous HeLa cells were grown to ca. 30% confluence and then transfected with control siRNA targeting EGFP (acting as control; A and B), *cdc25C* (C and D), or *cdc25A* (E and F). Twenty-four hours after transfection, cells were pulsed for 24 h with BrdU and then fixed. Subsequently, the transfected cells were costained for DNA (A, C, and E) and incorporated BrdU (B, D, and F). Scale bar, 10 μ m.

the broad specificity phosphatase substrate 3-OMFP principally because of its acute sensitivity. However, like pNPP, 3-OMFP presents the drawback of giving no indication about the nature of the *in vivo* substrate. Current experiments are underway to determine which proteins associate with *cdc25C* during S-phase with the objectives of determining the intracellular target activated at S-phase.

The marked increase in *cdc25C* protein levels and activity at the S-phase entry led us to examine whether there was functional requirement for this mitotic phosphatase as early as S-phase. For this we chose to interfere with *cdc25C* expression using antisense approaches. A well-documented problem with the use of antisense molecules is that nonspecific effects can cause artifacts (for review, see Branch, 1998). The antisense oligonucleotides were designed for stability and specificity with a length of ~20 base pairs, phosphorothioate modifications at their ends, and by avoiding any of the sequence motifs previously described to induce nonspecific antiproliferative effects. Furthermore, we used a number of different antisense oligonucleotides and vectors covering distinct regions of *cdc25C*, and all of them similarly affected entry into S-phase, whereas neither sense *cdc25C* nor antisense *cdc25B* molecules induced such effects. Because mammalian *cdc25* gene products display a high de-

gree of homology and *cdc25A* appears as an essential regulator of G1 progression and/or S-phase entry (Jinno *et al.*, 1994; Blomberg and Hoffmann, 1999; Sexl *et al.*, 1999), it was important to discount the possibility that *cdc25C* antisense molecules affected *cdc25A* nonspecifically. For this we used siRNA to specifically inhibit *cdc25C* expression (Elbashir *et al.*, 2001). SiRNA-induced suppression of *cdc25C* was accompanied by a block at G1/S in the presence of unchanged levels of *cdc25A* or B proteins, proving that only *cdc25C* was targeted but not *cdc25A* or B.

We also show that the effects of *cdc25C* antisense or siRNA molecules on G1-S transition could be specifically attributed to a lack of *cdc25C* phosphatase activity because microinjection of active *cdc25C* phosphatase was required to restore S-phase transit. This role of *cdc25C* is distinct from that of *cdc25A* since *cdc25A* levels were unaffected in *cdc25C*-depleted cells. In addition we have observed that, unlike siRNA to *cdc25C* that left levels of cyclin A unchanged, siRNA to *cdc25A* resulted in a strong reduction in cyclin A levels (Figure 4E), further supporting that *cdc25A* and C have distinct roles at S-phase and that in human cells, *cdc25A* cannot substitute for *cdc25C* at S-phase. Finally, we showed that *cdc25A* ablation like *cdc25C* also arrested cells before S-phase. This arrest was not due to nonspecific effects

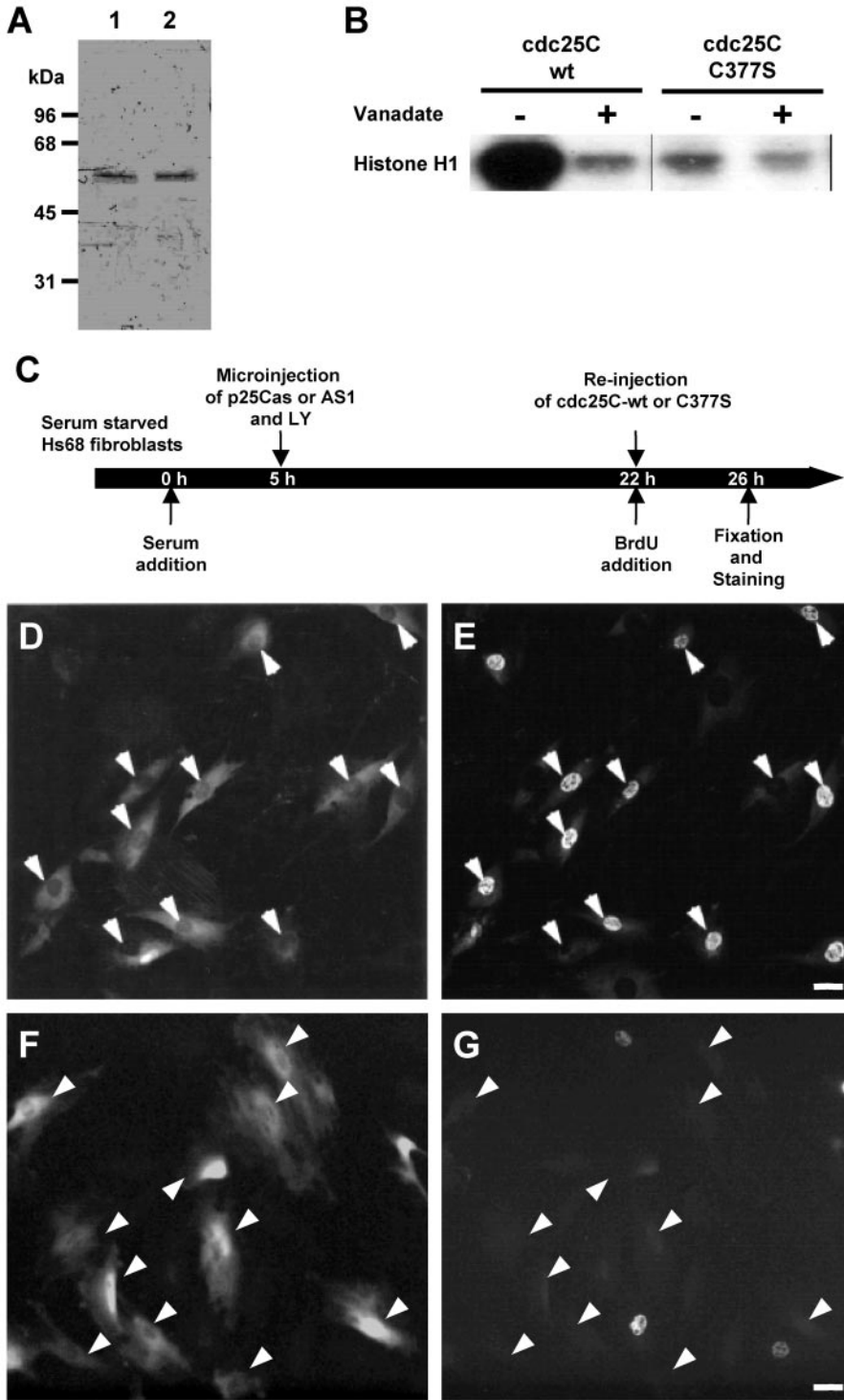


Figure 6. Active *cdc25C* restores S-phase in *cdc25C*-antisense cells. Wild-type *cdc25C* and mutant *cdc25C*-C377S were expressed in *E. coli* and purified to apparent homogeneity. (A) Approximately 0.5 μg of wild-type (lane 1) and C377S protein (lane 2) were separated on 10% SDS-PAGE. Purity was assessed by Coomassie Blue staining. The molecular masses of marker proteins in kilodaltons are indicated at the left. (B). To confirm the activity of purified *cdc25C* proteins, low-activity *cdc2*/cyclin B1 complexes were immunoprecipitated from early G2 HeLa cells and incubated with purified *cdc25C* WT or catalytically inactive *cdc25C*-C377S in the presence or absence of 2 mM sodium vanadate. Subsequently, histone H1 kinase activity was measured. Shown is a typical autoradiograph of histone H1 phosphorylation induced by the activation of *cdc2*. (C–G) As schematized in C Hs68 fibroblasts were synchronized by serum starvation. Five hours after serum readdition, cells were microinjected with p25Cas together with Lucifer Yellow (LY). Twenty-two hours after refeeding, injected cells were identified by the presence of LY in the cytoplasm and re-injected with active, recombinant *cdc25C*-wt (D and E), or inactive, recombinant *cdc25C*-C377S (F and G). Immediately after the second injection cells were further cultured in the presence of BrdU for 4 h. Cells were then fixed and stained for BrdU incorporation (E and G) and rabbit marker antibodies (D and F; included during the second injection). Arrowheads depict microinjected cells. Scale bars, 10 μm.

of siRNA because siRNA targeting *cdc25B* had no effect on S-phase entry while ablating *cdc25B* expression. A similar lack of effect was observed when cells were transfected with siRNA targeting human MEF2 (our unpublished results).

Previous studies have concluded that the principal *cdc25* phosphatase active at S-phase entry is *cdc25A* (Jinno *et al.*, 1994) with the two other isoforms being attributed roles at mitosis (Millar *et al.*, 1991; Gabrielli *et al.*, 1996; Lammer *et al.*,

Table 2. DNA synthesis in antisense-cdc25C injected cells following cdc25C protein microinjection.

| Microinjection | No. of cells re-injected | Number of cell positive for BrdU | % cells positive for BrdU |
|--|--------------------------|----------------------------------|---------------------------|
| Cdc25C-wt into antisense plasmid p25as cells | 22 | 18 | 82 |
| Cdc25C-wt into antisense oligo AS1 cells | 49 | 30 | 62 |
| Cdc25C-C377S into antisense oligo AS1 cells | 42 | 3 | 7 |

Background DNA synthesis levels was 96%. Hs68 fibroblasts were synchronised by serum starvation. Five hours after serum readdition cells were microinjected with p25Cas or oligonucleotide AS1 and Lucifer Yellow (LY). Twenty-two hours after refeeding, injected cells were identified by the presence of LY in the cytoplasm and re-injected with active cdc25C-wt, inactive cdc25C-C377S or cyclin A. Immediately after the second injection cells were further cultured in the presence of BrdU for 4 h. Cells were then fixed and stained for rabbit marker antibodies (for the number of cells re-injected) and BrdU incorporation (for cells that have entered S-phase). Total results from two independent experiments are shown. DNA synthesis in antisense-injected, but not re-injected, surrounding cells was 14% (for p25as) and 15% (for AS1).

1998). Importantly, antisense cdc25B or cdc25C oligonucleotides affected mitotic transit when microinjected into G2 cells (Lamb, N.J.C., unpublished result) demonstrating that they also inhibited previously described functions of these two phosphatases. A role of cdc25C during S-phase appears unprecedented, but a number of technical and/or functional reasons could explain why such a role may have been systematically overlooked in previous studies. First, cdc25C being required at two points in the cell cycle is more difficult to demonstrate than with a single cell cycle function, especially because most previous studies concentrated essen-

tially on mitosis using synchronized cells in S and G2 (Millar *et al.*, 1991; Hoffmann *et al.*, 1993; Strausfeld *et al.*, 1994; Gabrielli *et al.*, 1996). Second, our initial results of S-phase arrest after cdc25C depletion were obtained in Hs68 fibroblasts, microinjected early in G1 when they contained little or no cdc25C protein. In contrast, in HeLa cells low cdc25C levels were only observable during a short time in late G1 (Figures 1 and 2). We also show that cdc25C is degraded during the first 60 min after thymidine release, and this brief window may well have been overlooked in previous studies. Lastly, some of the multiple phosphorylation sites im-

Figure 7. Active cdc25C overcomes the block of DNA synthesis in siRNA-cdc25C-transfected cells. Asynchronously growing HeLa cells were transfected with siRNA duplexes targeting cdc25C. Thirty-six hours after transfection, cells were microinjected with either active cdc25C-wt protein (A, C, and E) or the inactive C377S mutant protein (B, D, and F). Cells were further cultured in the presence of BrdU for 4 h before fixation. Subsequently cells were costained for DNA (A and B), BrdU (C and D), and rabbit marker antibodies (E and F; included in the microinjection solution). Scale bar, 10 μ m.

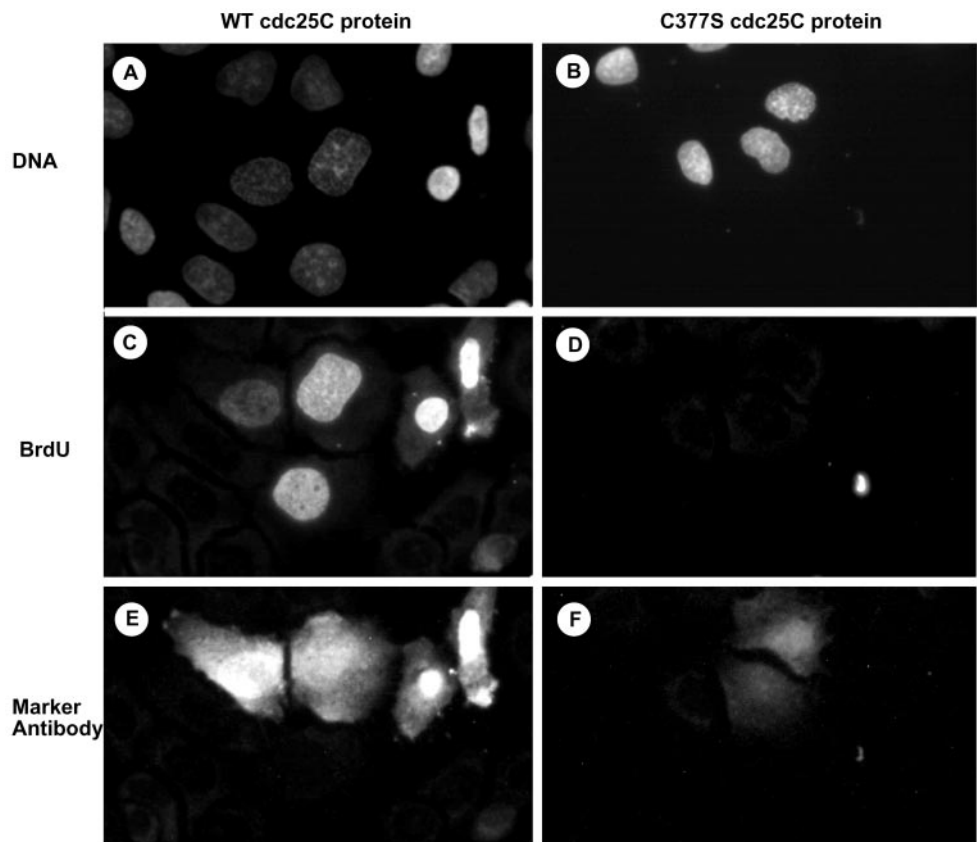


Table 3. DNA synthesis in *cdc25C* siRNA-transfected cells following *cdc25C* protein microinjection.

| Microinjection | No. of cells injected | Number of cell positive for BrdU | % of cells rescued |
|-------------------|-----------------------|----------------------------------|--------------------|
| Cdc25C-wt | 84 | 76 | 90 |
| Cdc25C-C377S | 77 | 5 | 6 |
| Marker antibodies | 90 | 5 | 5 |

Background level of DNA synthesis was 5%. Asynchronously growing HeLa cells were transfected with siRNA duplexes targeting *cdc25C*. Thirty-six hours after transfection, cells were microinjected with active *cdc25C*-wt protein, inactive C377S mutant protein, or microinjection marker antibodies alone. Cells were further cultured in the presence of BrdU for 4 h before fixation. Subsequently cells were co-stained for rabbit marker antibodies (for the number of cells injected) and BrdU (for the number of cells that have entered S-phase). Numbers represent mean values from four independent experiments.

plicated in the mitotic activation of *cdc25C* (Hoffmann *et al.*, 1993; Strausfeld *et al.*, 1994; Lammer *et al.*, 1998) may not be restricted to mitosis, because we have recently observed phosphorylation of some "mitotic" sites as early as S-phase using phosphorylation site-specific antibodies (our unpublished observations).

Our data imply that a significant difference must exist between mice and humans with respect to *cdc25C*. In mice, data from gene knockouts suggests that *cdc25C* has a non-essential and redundant role (Chen *et al.*, 2001). We have shown here that in both normal and transformed human cells, *cdc25C*, in addition to *cdc25A*, is required for entry into S-phase. These functions are clearly nonredundant because human cells blocked in the absence of *cdc25C* have wild-type levels of *cdc25A* and vice versa. Analysis of the protein product from the mouse *cdc25C* gene suggests that it differs significantly from its human counterpart. This is particularly notable with respect to the cytoplasmic anchoring region required for binding *cdc25C* to 14.3.3 and the putative NLS (amino acid region 200–250 in human *cdc25C*, absent in mouse *cdc25C*). In addition, key sites for cell cycle checkpoint kinase signaling cascades are also absent in mouse *cdc25C*. Murine *cdc25C* protein shows many similarities with the alternatively spliced forms of human *cdc25C* identified in human cancer cells (Bureik *et al.*, 2000; Wegener *et al.*, 2000). These forms are thought to promote deregulated proliferation due to the absence of key N-terminal regulatory sequences. It is therefore possible that in mice, the key functions of *cdc25* phosphatases have been taken over by *cdc25A* with *cdc25C* playing a dispensable role similar to the divergent forms seen in human cancers. In addition, there is also a possibility that, unlike the analysis in living organisms where the time scale after knock-out allows establishing compensatory mechanism(s), antisense and siRNA targeting studies leave no time for such compensation to develop.

The finding that *cdc25C* is specifically synthesized and required at S-phase, raises the question of what role it plays. Our data show that *cdc25C* activity is induced during S-phase with a peak coinciding closely with maximal DNA synthesis, suggesting that *cdc25C* functions in the course of DNA replication. However, very much as for cyclin A (Girard *et al.*, 1991), cells lacking *cdc25C* do not initiate S-phase because we did not even observe a punctate BrdU staining. There is accumulating evidence of cellular mechanisms to measure the presence of essential cell cycle components well before they are required. For instance centrosomes need to

be present in G1 for the cell to commit to S-phase (Hinchcliffe *et al.*, 2001). Therefore the lack of *cdc25C* could be detected by a sensor mechanism, such as the S-phase checkpoint. Our results also show that catalytically active *cdc25C* is required to enter S-phase, whereas inactive *cdc25C* polypeptide is not sufficient to relieve the S-phase block. Thus this checkpoint would rather measure phosphatase activity than the mere presence of protein. We are currently examining the status of the human checkpoint kinases in *cdc25C*-depleted cells with the objective of determining whether they are involved in detecting the absence of *cdc25C* activity. Most importantly, the requirement for active *cdc25C* to enter S-phase suggests a role in the activation of a cdk-cyclin complex, and future work will concentrate on the identification of such a complex. Cdk2 could be a candidate molecule: its activating role in S-phase induction is well documented (Ekholm and Reed, 2000). However, we did not find significant changes in either cdk2 activity or its phosphorylation status when cells were depleted of *cdc25C* by siRNA. *Cdc25A* has been implicated in the activation of cdk2 (Blomberg and Hoffmann, 1999), but a mechanism involving direct dephosphorylation of cdk2 by *cdc25A* has been challenged (Sexl *et al.*, 1999). *Cdc25A* and/or C may still dephosphorylate a specific subpopulation of cdk2 for a precise function and such subtle changes may not be readily observable with the currently available techniques. Yet another possible function of *cdc25C* upstream of cyclin A synthesis seems unlikely because cyclin A is solely regulated by transcription (Pines and Hunter, 1990), and *cdc25C* transcription underlies the same mechanism. Alternatively, *cdc25C* could modulate the half-life of cyclins or other cell cycle proteins at S-phase. Indeed the finding that *cdc25C* is specifically lost in cycling late G1 HeLa cells suggests that a degradation mechanism plays a key function in modulating G1 to S-phase progression. A recent study has shown that hEmi1, originally identified as an inhibitor of APC20 ubiquitination in mitosis, is also transcriptionally induced at S-phase entry where it inhibits APCdh1, and consequently promotes cyclin A accumulation (Hsu *et al.*, 2002). It will be interesting to investigate whether *cdc25C* is implicated in this mechanism either as a target for the APC or in modulating the activation of hEmi1.

In conclusion, we demonstrate that like *cdc25A*, but unlike *cdc25B*, *cdc25C* is necessary for G1-S transition in transformed and nontransformed human cells. This previously unidentified function sheds new light on the biology of

cdc25 phosphatases, like a number of recent reports that implicate cdc25B in mitotic induction (Nishijima *et al.*, 1997; Lammer *et al.*, 1998; Karlsson *et al.*, 1999) or cdc25A during G2/M transition (Mailand *et al.*, 2002). Taking into account the increasing number of cdc25 splice variants found in mammals (Baldin *et al.*, 1997; Bureik *et al.*, 2000; Wegener *et al.*, 2000), the original roles assigning respectively cdc25A, B, and C to G1/S, S/G2, and G2/M progression and the proposed substrates for these different dual specificity phosphatases need to be reexamined. It appears feasible that more than one cdc25 phosphatase is implicated in the control of a given cell cycle transition and that they are imbedded in several parallel and hierarchical networks.

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