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Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6

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ABSTRACT We have characterized HsCdc6, a human protein homologous to the budding yeast Cdc6p that is essential for DNA replication. We show that, unlike Cdc6p, the levels of HsCdc6 protein remain constant throughout the cell cycle in human cells. However, phosphorylation of HsCdc6 is regulated during the cell cycle. HsCdc6 is an excellent substrate for Cdk2 *in vitro* and is phosphorylated *in vivo* at three sites (Ser-54, Ser-74, and Ser-106) that are phosphorylated by Cdk2 *in vitro*, strongly suggesting that HsCdc6 is an *in vivo* Cdk substrate. HsCdc6 is nuclear in G_1, but translocates to the cytoplasm at the start of S phase via Crm1-dependent export. An HsCdc6A1A2A3 mutant, which mimics unphosphorylated HsCdc6, is exclusively nuclear, and its expression inhibits initiation of DNA replication. An HsCdc6E1E2E3 mutant, which mimics phosphorylated HsCdc6, is exclusively cytoplasmic and is not associated with the chromatin/nuclear matrix fraction. Based on these results, we propose that phosphorylation of HsCdc6 by Cdks regulates DNA replication of at least two steps: first, by promoting initiation of DNA replication and, second, through nuclear exclusion preventing DNA rereplication.

In all eukaryotic cells, DNA replication is a tightly regulated process that is strictly coupled to the progression of the cell cycle. It occurs only during S phase, and initiation of DNA replication occurs at discrete chromosomal locations (replication origins). When DNA replication is initiated, the cell must ensure that all its genome is replicated and that the replication of every DNA section occurs once and only once during the cell cycle. In the budding yeast, Cdc6p plays a unique role in regulating DNA replication. It is essential for initiation of DNA replication and required for assembly and maintenance of the prereplication complexes (pre-RCs) at replication origins (1, 2). In contrast to the origin recognition complex (ORC) and minichromosome maintenance (Mcm) proteins, Cdc6p is expressed only in G_1 phase of the *Saccharomyces cerevisiae* cell cycle (3, 4). Cdc6p and its *Schizosaccharomyces pombe* homologue Cdc18 physically interact with ORC, and Cdc6p is required for the loading of Mcm proteins at the replication origins in G_1 (5, 6). Cdc6p is a nucleotide-dependent loading factor related to the eukaryotic and prokaryotic clamp loaders, such as PCNA (7, 8). Both Cdc6p and Cdc18 also are associated with Cdks *in vivo* and can be phosphorylated by Cdks *in vitro* (9, 10). It is thought that Cdk phosphorylation of Cdc6p and Cdc18 triggers their degradation through a ubiquitination-mediated protein-degradation pathway (11–13). A gain-of-function Cdc6p mutant displays promiscuous initiation of DNA replication and promotes constant Mcm proteins association with chromatin throughout the cell cycle (14). Overexpression of a Cdc18 mutant that cannot be phosphorylated by Cdks *in vitro* causes greater DNA overreplication in *Schizosaccharomyces pombe* than expression of wild-type Cdc18 (13, 15). Taken together, these findings indicate that Cdc6p/Cdc18 is a key player in regulating assembly and disassembly of pre-RCs on origins in the yeast. Cdc6p/Cdc18 can promote formation of pre-RCs only during G_1 when it is expressed and Cdks are inactive. Once replication begins, Cdc6p/Cdc18 is destroyed rapidly by ubiquitination-mediated protein degradation, and the lack of Cdc6p/Cdc18 in S to G_2/M ensures that Mcm proteins cannot bind to origins during these stages of the cell cycle, thereby preventing DNA rereplication.

The elucidation of processes involved in initiation of DNA replication has been slower in higher eukaryotes including human cells, in part, because the sequence(s) characteristic of replication origins are only just being defined (16) and identification of replication proteins is less complete. Nonetheless, the results obtained from the *Xenopus* cell-free replication system in which "licensing" for DNA replication depends on XORC, XCdc6, and XMcm proteins suggest that initiation of the DNA-replication pathway is conserved during evolution (17–21). Moreover, the human homologue of Cdc6, HsCdc6 (22, 23), is essential for replication in human cells (24), and added recombinant Cdc6 protein causes premature entry into S phase in a mammalian cell-free system (25).

To learn more about how initiation of DNA replication is regulated in mammalian cells, we have examined how HsCdc6 protein expression is regulated during the cell cycle and its functional role in controlling initiation of DNA replication in human cells. Unlike Cdc6p and Cdc18 in the yeast, the level of HsCdc6 protein is not regulated during the cell cycle. However, HsCdc6 is phosphorylated in a cell cycle-regulated fashion at sites phosphorylated by Cdks *in vitro*. We provide direct evidence that phosphorylation of HsCdc6 by Cdks is required for initiation of DNA replication and prevents its reassociation with chromatin via Crm1-dependent export from the nucleus. Based on these results, we propose that phosphorylation of HsCdc6 by Cdks regulates DNA replication of at least two steps: first, by promoting initiation of DNA replication and, second, through nuclear exclusion preventing DNA rereplication in human cells.

MATERIALS AND METHODS

Cloning and Plasmid Construction. An ≈240-bp human HsCdc6 cDNA fragment was isolated from HeLa cells by PCR amplification by using degenerate oligonucleotide primers specific to regions of high amino acid sequence similarity shared between the *Saccharomyces cerevisiae* Cdc6p (residues 108–115 and 219–225) and *Schizosaccharomyces pombe* Cdc18 protein (residues 199–206 and 282–288). The 5′ and 3′ primers were 5′-CCCGGAATTCGGA/G/C/TGCA/G/C/TCCA/

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Abbreviations: pre-RCs, prereplication complexes; ORC, origin recognition complex; Mcm protein, minichromosome maintenance protein; PAA, phosphoamino acid analysis; 2D, two-dimensional; LMB, leptomycin B; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

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G/C/TGGA/G/C/TACA/G/C/TGGA/G/C/TAAA/GAC-3′ and 5′-CCGCGGATCCA/GTCCATC/TTCA/GTCA/G/C/TAAA/G/C/TACA/G/C/TA-3′, respectively. The cDNA fragment was used as a probe to obtain a full-length HsCdc6 cDNA from a HeLa cDNA library constructed in λGEX5 (26). Conceptual translation of the full-length HsCdc6 cDNA reveals a 560-deduced-aa ORF with predicted size of 62 kDa. Recently, *Xenopus* and human proteins highly related to Cdc6p also have been identified independently by several other research groups, and the human Cdc6 sequences are identical to HsCdc6 (18, 22, 23).

To generate glutathione *S*-transferase (GST)-HsCdc6 or its deletion mutants, ΔN and ΔC, the corresponding sequences of HsCdc6 (residues 1–560), ΔN-HsCdc6 (residues 142–560), or ΔC-HsCdc6 (residues 1–142) were amplified by PCR using Pfu polymerase (Stratagene) and subcloned into the *Bam*HI and *Xho*I sites of pGEX-KG vector. To generate point mutants (A1, A2, A3, A1A2, and A1A2A3) of GST-HsCdc6 fusion proteins, in which Ser-54, Ser-74, Ser-106, Ser-54 and Ser-74, or all three were replaced with alanines, pGEX-KG HsCdc6-A1, -A2, -A3, -A1A2, and -A1A2A3 were constructed by the quick-change mutagenesis method according to the manufacturer's description (Stratagene). To generate mammalian expression constructs, HsCdc6 and its mutants were subcloned into *Bgl*II and *Xho*I sites of the cytomegalovirus-based pCS3 mammalian expression vector, which provides an N-terminal $(Myc)_6$ epitope tag (27). The pCS3HsCdc6E1E2E3 mutant, in which Ser-54, Ser-74, and Ser-106 were replaced by glutamates, was generated by the quick-change mutagenesis method. To generate recombinant retrovirus expression constructs, HsCdc6, HsCdc6A1A2A3, and HsCdc6E1E2E3 were subcloned into the *Xho*I-*Bam*HI sites of a retroviral expression vector pCLXSN-GFP, which provides an N-terminal green fluorescent protein (GFP) tag (W.J., unpublished data). All PCR and mutagenesis products were verified by DNA sequencing.

Antibodies and Immunofluorescence Staining. Polyclonal antibodies were developed in rabbits against a synthetic polypeptide, CEIEHALKDKALIGNILATGL, corresponding to the C-terminal residues 540–559 of HsCdc6, with an appended N-terminal Cys for coupling. The antibodies were purified from the serum of rabbits 5956 and 5957 by using a GST-HsCdc6 fusion protein affinity column as described (27). The 9E10 mAb was used for immunoblotting and immunostaining of the Myc-tagged proteins. The anti-BrdUrd mAb used for immunostaining in BrdUrd incorporation experiments and the anti-histone mAb for immunoblotting experiments were purchased from Accurate Scientific (Westbury, NY; clone-BU1/75) and Boehringer Mannheim (clone H11–4), respectively. Standard protocols were used for immunofluorescence staining as described (27).

Cell Culture, Retrovirus Infection, Cell Cycle Synchronization, *in Vivo* Metabolic Labeling, Phosphoamino Acid Analysis (PAA), and Two-Dimensional (2D) Tryptic Phosphopeptide Mapping. 293, HeLa, and U2OS cell lines and normal human neonatal foreskin fibroblasts, HSF8 (W.J., unpublished data), were cultured in DMEM with 10% FCS. GFP-HsCdc6 retroviruses were generated and used to infect early-passage (passage 4–8) HSF8 cells as described (28). Cell cycle synchronization and flow cytometric analysis were performed as described previously (29). For *in vivo* metabolic labeling, synchronous cells at G_2/M, M/G_1, G_1/S, and S/G_2 were washed once with phosphate-free medium and then incubated with fresh phosphate-free medium supplemented with 10% dialyzed calf serum and 2 mCi/ml [^{32}P]orthophosphate (for G_1/S phase cells also containing 2 mM thymidine and for G_2/M phase cells also containing 20 ng/ml nocodazole) for 4 h. PAA and 2D analysis of tryptic phosphopeptides were performed as described (30). To determine DNA replication *in vivo*, HFS8 cells that expressed GFP or GFP-HsCdc6 proteins were grown on glass coverslips and arrested in G_0 by incubating them for 3 days in DMEM with 0.1% FCS. They were stimulated to enter the cell cycle by adding fresh medium with 20% FCS in the presence of 10 μM BrdUrd. Cell cycle progression was monitored by measuring BrdUrd incorporation (see preceding paragraph).

Protein Extraction, Chromatin and Nuclear Matrix Fractionation, Immunoprecipitation, Immunoblotting, and *in Vitro* Protein Kinase Assay. Standard protocols for protein extraction, immunoblot analysis, and protein kinase assay were used as described (27, 29). The chromatin/nuclear matrix fractionation assay was performed as described (31). Briefly, U2OS cells were washed three times with ice-cold PBS and then lysed with 1 ml modified CSK buffer (10 mM Pipes, pH 6.8/100 mM NaCl/300 mM sucrose/1 mM $MgCl_2$/1 mM EGTA/1 mM DTT/1 mM PMSF/10 units/ml aprotinin/20 μg/ml leupeptin/50 μM Na_3VO_4/5 mM β-glycerophosphate/5 mM NaF/2 mM ATP/0.5% Triton X-100) for 10 min. After low-speed centrifugation (3,000 rpm, 3 min at 4°C), the nuclei were extracted twice more with 1 ml of ice-cold modified CSK buffer for 10 min on ice. The soluble and nuclear fractions were mixed with an equal volume of 2× sample buffer, separated by SDS/PAGE, and then immunoblotted with 9E10 mAb and anti-histone mAb, respectively.

RESULTS AND DISCUSSION

Expression and Phosphorylation of HsCdc6 During the Cell Cycle. To study the function of HsCdc6, we examined the expression levels of HsCdc6 protein during the cell cycle in human cells. U2OS osteosarcoma cells were synchronized at G_2/M by thymidine/nocodazole treatment, and the amount of HsCdc6 protein in the cells at different times after release from the thymidine/nocodazole block was analyzed by immunoblotting with affinity-purified anti-HsCdc6 antibodies. The expression levels of the 62-kDa HsCdc6 protein remained constant, irrespective of the proportion of cells in specific phases of the cell cycle (Fig. 1*A*). Similar results also were obtained with synchronized HeLa cells and with cells synchronized by blocking and releasing from G_1/S (data not shown). Thus, unlike the yeast Cdc6p and Cdc18, which are expressed only in G_1, HsCdc6 levels did not fluctuate during the cell cycle in synchronous human cells. This raised the possibility that the activity of HsCdc6 might be regulated through posttranslational modification(s) during the cell cycle. Therefore, we tested whether HsCdc6 is phosphorylated and whether phosphorylation of HsCdc6 is regulated during the cell cycle. U2OS cells were metabolically labeled with [^{32}P]orthophosphate for 4 h at various stages of the cell cycle, and HsCdc6 protein was immunoprecipitated with affinity-purified anti-HsCdc6 antibodies. Although similar amounts of HsCdc6 protein were immunoprecipitated from ^{32}P-labeled cells at different stages of the cell cycle as determined by immunoblotting, phosphorylation of HsCdc6 protein clearly was regulated during the cell cycle (Fig. 1*B*). Phosphorylation of HsCdc6 was low in M/G_1 to G_1/S, but increased during S/G_2 to G_2/M. This result indicated that HsCdc6 is phosphorylated and that phosphorylation of HsCdc6 is regulated in a cell cycle-dependent manner.

HsCdc6 Is Phosphorylated *in Vivo* at Ser-54, Ser-74, and Ser-106, Which Are Phosphorylated by Cdks *in Vitro*. Because HsCdc6 has three consensus Cdk phosphorylation sites, Ser-54 (SPRK), Ser-74 (SPSK), and Ser-106 (SPPK), in its N-terminal region and phosphorylation of HsCdc6 is regulated during the cell cycle, we examined whether HsCdc6 was a Cdk substrate. HsCdc6 was expressed as a GST-fusion protein in *E. coli* and purified by glutathione-agarose chromatography. GST-HsCdc6 was incubated with several purified, baculovirus-expressed cyclin-Cdk complexes in the presence of [γ-^{32}P]ATP (Fig. 2*A*). When compared with GST-Rb, the best-

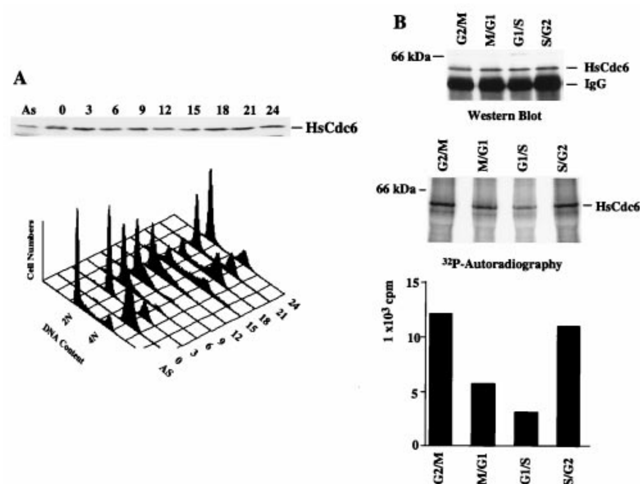

FIG. 1. (*A*) Expression and phosphorylation of HsCdc6 during the cell cycle. U2OS cells were synchronized by a "thymidine/nocodazole" block at G_2/M phase and then released for different times (h) as indicated. Fifty micrograms of cell lysates from the indicated time point were subjected to SDS/PAGE, transferred to Immobilon-P membrane, and then immunoblotted with affinity-purified anti-HsCdc6 antibodies (*Upper*). Cell cycle position of U2OS cells used in the immunoblotting analysis at indicated time points (*Upper*) was determined by flow cytometry (*Lower*). (*B*) U2OS cells were synchronized at various stages of the cell cycle as indicated and *in vivo* labeled with [^{32}P]orthophosphate for a period of 4 h. HsCdc6 was immunoprecipitated by using anti-HsCdc6 antibodies. After washing, the immunoprecipitates were subjected to SDS/PAGE and visualized by autoradiography (*Middle*). The bands containing ^{32}P-labeled HsCdc6 proteins were quantified by a PhosphorImager (*Bottom*). The amounts of immunoprecipitated HsCdc6 protein were determined by using anti-HsCdc6 antibodies for immunoblotting (*Top*).

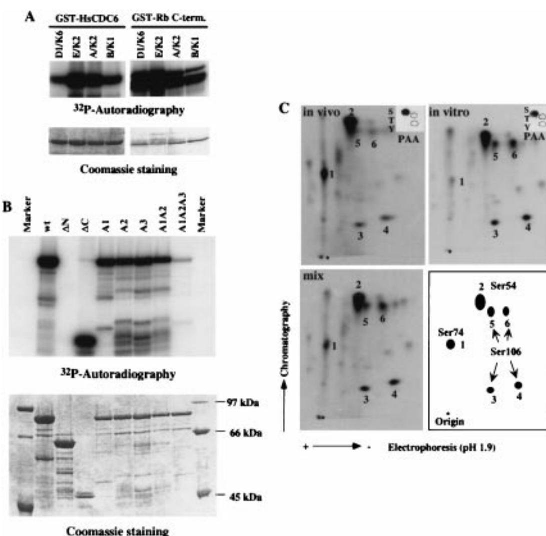

FIG. 2. HsCdc6 is a Cdk substrate. (*A*) Substrate specificity of cyclin-Cdk complexes against GST-HsCdc6 in comparison with a known substrate protein, GST-Rb C terminus (residues 768–928). One to 2 μg GST-HsCdc6 or GST-Rb was incubated with purified baculovirus-expressed cyclin D1-Cdk6 (D1/K6), cyclin E-Cdk2 (E/K2), cyclin A-Cdk2 (A/K2), or cyclin B1-Cdc2 (B/K1) in the presence of [γ-^{32}P]ATP. Proteins were resolved by SDS/PAGE and visualized by autoradiography (*Upper*) or Coomassie blue staining (*Lower*). (*B*) The GST-HsCdc6wt and its mutant proteins described in *Materials and Methods* were incubated with cyclin E-Cdk2 in the presence of [γ-^{32}P]ATP. Proteins were resolved by SDS/PAGE and visualized by autoradiography (*Upper*) or Coomassie blue staining (*Lower*). (*C*) ^{32}P-labeled immunoprecipitated HsCdc6 protein from cells in S/G_2 described in Fig. 1*B* (*in vivo*) and GST-HsCdc6 protein phosphorylated by cyclin E-Cdk2 described in *A* (*in vitro*) were eluted from SDS/polyacrylamide gels and then digested with trypsin. The resulting phosphopeptides were separated by electrophoresis (pH 1.9 buffer) in the horizontal dimension (anode on the left) and chromatography (isobutyric acid buffer) in the vertical dimension. Shown are 2D tryptic phosphopeptide maps of: *in vivo*, HsCdc6 from *in vivo* ^{32}P-labeled G_2/M cells (2,000 cpm); *in vitro*, GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* (2,000 cpm); mix, mix of *in vivo* (1,000 cpm) and *in vitro* (1,000 cpm) and the schematic map. The major phosphopeptides, 1–6, are labeled. PAA of *in vivo* ^{32}P-labeled HsCdc6 and GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* were performed, and radioactive phosphoamino acids were visualized by autoradiography (*Insets*).

characterized physiological Cdk substrate, GST-HsCdc6 was phosphorylated efficiently by cyclin E-Cdk2, cyclin A-Cdk2, and, to a lesser extent, by cyclin B-Cdc2, but poorly by cyclin D1-Cdk6. Thus, HsCdc6 was an excellent substrate for Cdk2 *in vitro*. To determine whether the three Cdk consensus phosphorylation sites in HsCdc6 could serve as Cdk phosphorylation sites, we generated several GST-HsCdc6 mutants (see *Materials and Methods*). In contrast to GST-HsCdc6wt and its other mutants that could be phosphorylated efficiently by cyclin E-Cdk2 *in vitro*, GST-HsCdc6 mutants, ΔN, in which the N-terminal 142 aa of HsCdc6 including all three consensus Cdk phosphorylation sites had been deleted, and A1A2A3, in which the consensus Cdk phosphorylation sites Ser-54, Ser-74, and Ser-106 were substituted by alanines, were poorly, if at all, phosphorylated by cyclin E-Cdk2 (Fig. 2*B*). PAA indicated that GST-HsCdc6 was phosphorylated *in vitro* exclusively on Ser by the cyclin-Cdk complexes. 2D tryptic phosphopeptide-mapping analysis of GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* revealed six major phosphopeptides (spots 1–6) (Fig. 2*C*). Comparison of 2D tryptic phosphopeptide maps of GST-HsCdc6wt and its mutants demonstrated that phosphopeptides, spot 2, spot 1, and spots 3–6 on the map of GST-HsCdc6wt contained Ser-54, Ser-74, and Ser-106, respectively (Fig. 2*C* and data not shown). Therefore, we conclude that Ser-54, Ser-74, and Ser-106 in HsCdc6 are the major cyclin-Cdk phosphorylation sites *in vitro*. To determine phosphorylation sites of HsCdc6 *in vivo*, PAA and 2D tryptic phosphopeptide-mapping analyses of the endogenous ^{32}P-labeled immunoprecipitated HsCdc6 from cells in S/G_2 in Fig. 1*B* were performed and then compared with those of GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* (Fig. 2*C*). As with GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro*, HsCdc6 was phosphorylated exclusively on Ser *in vivo* (Fig. 2*C*). The 2D tryptic phosphopeptide map of *in vivo* ^{32}P-labeled HsCdc6 was virtually identical to that of GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* (Fig. 2*C*). The six major phosphopeptides (spots 1–6) from *in vivo* ^{32}P-labeled HsCdc6 comigrated with phosphopeptides (spots 1–6) of GST-HsCdc6 phosphorylated by cyclin E-Cdk2 *in vitro* when a mixture was analyzed (Fig. 2*C*). These results indicated that HsCdc6 is phosphorylated at Ser-54, Ser-74, and Ser-106 *in vivo*. This conclusion was confirmed further by an *in vivo* labeling experiment with $(Myc)_6$-tagged HsCdc6A1A2A3 mutant. *In vivo* phosphorylation of $(Myc)_6$-HsCdc6A1A2A3 protein transiently expressed in U2OS cells was not detected (data not shown). Thus, because phosphorylation of HsCdc6 is regulated during the cell cycle and HsCdc6 is phosphorylated *in vivo* at the sites that are phosphorylated by Cdk2 *in vitro*, we conclude that HsCdc6 is an *in vivo* physiological Cdk substrate.

Phosphorylation of HsCdc6 Regulates Its Subcellular Localization. Recent studies show that proteins involved in regulating DNA replication, such as Cdc6p and Mcms, are associated with chromatin, presumably with replication origins (5, 31–33). To elucidate the functional consequences of HsCdc6 phosphorylation by Cdks *in vivo*, we examined chromatin association of HsCdc6 and its phosphorylation-site mutants in human cells. To generate a mutant HsCdc6 protein

that mimics Cdk-phosphorylated HsCdc6, we made $(Myc)_6$-HsCdc6E1E2E3, in which Ser-54, Ser-74, and Ser-106 were replaced by glutamates. $(Myc)_6$-HsCdc6wt, $(Myc)_6$-HsCdc6A1A2A3, and $(Myc)_6$-HsCdc6E1E2E3 were transiently expressed in U2OS cells, and 10% of the cells were lysed in sample buffer before separation by SDS/PAGE and immunoblotting with 9E10 mAb to verify the expression levels of these proteins (Fig. 3*A*). The remaining 90% of the cells were used for subcellular fractionation (31). Proteins in the soluble and nuclear/chromatin fractions were resolved by SDS/PAGE and then immunoblotted with 9E10 mAb or the antibody against chromatin-associated protein histone H1. Whereas histone H1 was detected only in the nuclear/chromatin fraction, $(Myc)_6$-HsCdc6wt and $(Myc)_6$-HsCdc6A1A2A3 proteins were detected in both soluble and nuclear/chromatin fractions. In contrast, $(Myc)_6$-HsCdc6E1E2E3 protein was detected only in the soluble fraction (Fig. 3*B*). This result indicated that a proportion of wild-type (wt) and A1A2A3 mutant HsCdc6 were associated with chromatin/nuclear matrix, whereas the E1E2E3 mutant, which mimics HsCdc6 in its phosphorylated state, was not associated with chromatin/nuclear matrix.

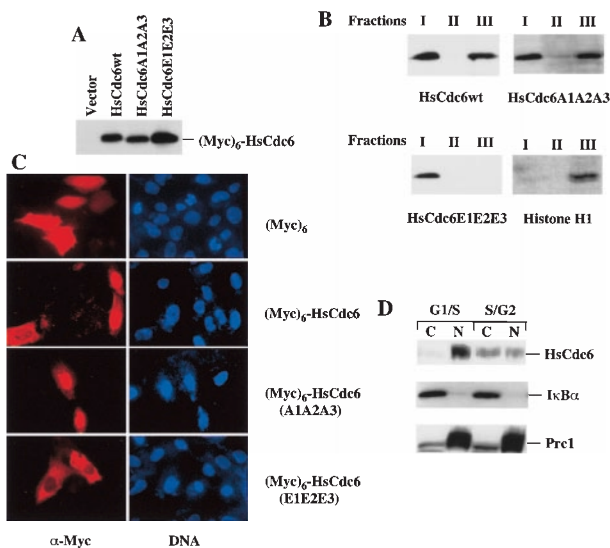

FIG. 3. Chromatin/nuclear matrix association and subcellular localization of $(Myc)_6$-HsCdc6wt, $(Myc)_6$-HsCdc6A1A2A3, and $(Myc)_6$-HsCdc6E1E2E3. (*A*) U2OS cells were transiently transfected with DNA constructs expressing $(Myc)_6$-HsCdc6wt, $(Myc)_6$-HsCdc6A1A2A3, $(Myc)_6$-HsCdc6E1E2E3, and control, $(Myc)_6$. Lysates from indicated transfected cells were subjected to SDS/PAGE and then analyzed by immunoblotting with the 9E10 mAb. (*B*) Cells expressing indicated proteins in *A* were subjected to chromatin/nuclear matrix fractionation. The soluble fraction from the first extraction (I), the soluble fraction from the third extraction (II), and the chromatin/nuclear matrix fraction (III) from the indicated cells were subjected to SDS/PAGE and then analyzed by immunoblotting with either the 9E10 mAb or anti-histone H1 mAb. (*C*) U2OS cells were grown on glass coverslips and transiently transfected with DNA constructs expressing indicated proteins as in *A*. After fixation, samples were incubated with the 9E10 mAb. Immunofluorescence staining was performed with Texas red-conjugated goat anti-mouse antibodies, and nuclei were visualized with Hoechst dye 33258. Fluorescent images were obtained by using a fluorescence microscope. (*D*) Subcellular localization of endogenous HsCdc6 during the cell cycle. HeLa cells were synchronized by a "double thymidine" block in G_1/S phase and then released into S/G_2 phase in fresh medium for a period of 6 h. Subcellular fractionation was performed as described (42, 43). Equal amounts of cytoplasmic and nuclear proteins extracted from G_1/S and S/G_2 cells were subjected to SDS/PAGE and then analyzed by immunoblotting with anti-HsCdc6 antibodies (*Top*), anti-IκBα (a cytoplasmic protein) antibodies [*Middle* (42)], or anti-Prc1 (a nuclear protein) antibodies [*Bottom* (29)], respectively.

There are two possible reasons why the HsCdc6E1E2E3 mutant protein is not associated with chromatin nuclear matrix: (*i*) HsCdc6E1E2E3 is unable to associate with chromatin/nuclear matrix but remains in the nucleus and (*ii*) HsCdc6E1E2E3 is relocalized from nucleus to cytoplasm. To distinguish between these two possibilities, we performed indirect immunofluorescent microscopy with the 9E10 mAb to determine the subcellular localization of wt and mutant HsCdc6 proteins in U2OS cells expressing these proteins. Representative photomicrographs are shown in Fig. 3*C*. Consistent with our published finding (27), immunofluorescent staining was detected in both the cytoplasm and nuclei of cells expressing 23-kDa $(Myc)_6$ tag polypeptide alone, indicating that the $(Myc)_6$ tag polypeptide was not localized in any specific subcellular compartment. In contrast, immunofluorescent staining was detected either in the nuclei or in the cytoplasm of cells expressing $(Myc)_6$-HsCdc6wt protein, i.e., $(Myc)_6$-HsCdc6 protein is in the nuclei of 50% of $(Myc)_6$-HsCdc6 positive cells and in the cytoplasm of the other 50% positive cells. However, immunofluorescent staining was detected exclusively in the nuclei of cells expressing $(Myc)_6$-HsCdc6A1A2A3 protein and exclusively in the cytoplasm of cells expressing $(Myc)_6$-HsCdc6E1E2E3 protein. When U2OS cells that expressed $(Myc)_6$-HsCdc6wt were synchronized at G_1, G_1/S, S/G_2, and G_2/M phases of the cell cycle after release from a double-thymidine or a thymidine/nocodazole block, immunofluorescent staining was detected mainly in the nuclei of cells in G_1 and G_1/S and in the cytoplasm of cells in S/G_2 and G_2/M (data not shown). Because the anti-HsCdc6 antibodies were not good enough for immunostaining, we performed a subcellular fractionation experiment in which subcellular localization of endogenous HsCdc6 protein was analyzed by immunoblotting with anti-HsCdc6 antibodies. Endogenous HsCdc6 protein levels were detected mainly in the nuclear fraction when HeLa cells were synchronized in G_1/S. After cells were released into S/G_2, HsCdc6 levels increased in the cytoplasmic fraction and decreased in the nuclear fraction (Fig. 3*D*).

Taken together, these results indicated that phosphorylation of HsCdc6 by Cdks during the cell cycle regulates its subcellular localization and that subcellular redistribution of phosphorylated HsCdc6 from nucleus to cytoplasm prevents its reassociation with the chromatin/nuclear matrix. Prior work had indicated that HsCdc6 subcellular localization is cell cycle-regulated (23). Moreover, while this paper was under review, Helin and colleagues (34) reported that phosphorylation of Ser-54, Ser-74, and Ser-106 in HsCdc6 regulates its subcellular localization in a manner identical to that observed here and that the physiological Cdk for HsCdc6 is likely to be cyclin A-Cdk2 rather than cyclin E-Cdk2 based on its ability to bind HsCdc6 and to elicit nuclear export (34).

Nuclear Export of Phosphorylated HsCdc6 Is Exportin 1 (Crm1)-Dependent. We next determined what mediates redistribution of phosphorylated HsCdc6 from the nucleus to the cytoplasm. To this end, we generated N-terminal GFP-tagged HsCdc6wt, HsCdc6A1A2A3, and HsCdc6E1E2E3 recombinant retroviruses. HSF8, human primary foreskin fibroblasts, were transiently infected with expression vectors for GFP-HsCdc6, GFP-HsCdc6A1A2A3, GFP-HsCdc6E1E2E3, and GFP alone by retrovirus-mediated transduction. Analysis of GFP fluorescence under a fluorescent microscope demonstrated that the subcellular localizations of GFP-HsCdc6wt, GFP-HsCdc6A1A2A3, and GFP-HsCdc6E1E2E3 mutant proteins in these cells were virtually identical to that of $(Myc)_6$-tagged HsCdc6 proteins expressed in U2OS cells (Fig. 4*A*). Recent studies have shown that export of many proteins from the nucleus to the cytoplasm is mediated mainly by an export receptor, exportin 1 (Crm1) (35–37). Crm1 binds to its cargoes in the nucleus in the presence of a small nuclear GTPase protein, RanGTP. After the RanGTP-Crm1-cargo complex is

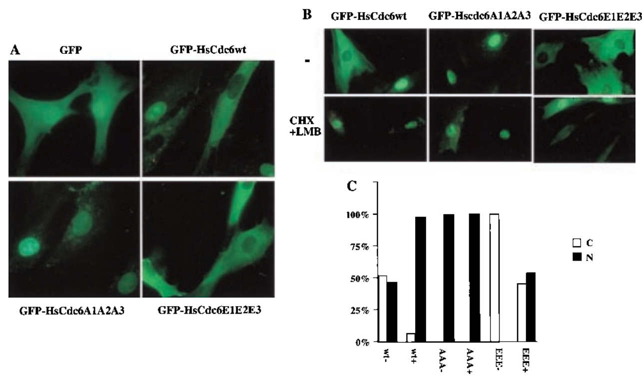

FIG. 4. Subcellular localization of GFP-HsCdc6 and its mutants upon LMB treatment. (*A*) GFP, GFP-HsCdc6wt, GFP-HsCdc6A1A2A3, and GFP-HsCdc6E1E2E3 were transiently expressed in HSF8 cells by retrovirus-mediated transduction for 24 h. After fixation, GFP fluorescent images were obtained by using a fluorescence microscope. (*B*) HSF8 cells expressing the indicated GFP-HsCdc6 proteins were treated with or without cycloheximide (CHX, 50 μg/ml) and LMB (20 nM) for 4 h. After fixation, GFP fluorescent images were obtained by using a fluorescence microscope. (*C*) Summary of quantitative results from *B*, in which $\approx$100 cells expressing the indicated GFP-HsCdc6 proteins treated with (+) or without (−) CHX+LMB were counted. Solid bars represent the percentage of GFP-HsCdc6 proteins in the nucleus, and open bars represent the percentage of GFP-HsCdc6 proteins in the cytoplasm.

translocated from the nucleus to the cytoplasm, the GTP in the complex is hydrolyzed, causing the cargo to dissociate from Crm1. A cytotoxin, leptomycin B (LMB), specifically blocks formation of the RanGTP-Crm1-cargo complex and, thus, inhibits Crm1-dependent protein export (35–38). To determine whether cytoplasmic localization of phosphorylated HsCdc6 is mediated by Crm1-dependent nuclear export, HSF8 cells expressing GFP-HsCdc6, GFP-HsCdc6A1A2A3, GFP-HsCdc6E1E2E3, or GFP alone were treated with 20 nM LMB in the presence of the protein synthesis inhibitor cycloheximide. LMB did not affect the subcellular localization of GFP or GFP-HsCdc6A1A2A3 in HSF8 cells. In contrast, LMB strongly inhibited cytoplasmic localization of GFP-HsCdc6 and GFP-HsCdc6E1E2E3 (Fig. 4*B*). These results demonstrate that cytoplasmic localization of phosphorylated HsCdc6 is mediated by Crm1-dependent nuclear export and that this nuclear export requires phosphorylation of HsCdc6 by Cdk. Analogous Cdk phosphorylation-dependent nuclear export recently has been reported for the yeast Pho4 transcription factor (39).

Phosphorylation of HsCdc6 Is Required for Initiation of DNA Replication. If phosphorylation of HsCdc6 by Cdks plays an important role in regulating DNA replication, ectopic expression of HsCdc6A1A2A3, a mutant that cannot be phosphorylated by Cdks nor exported from the nucleus to the cytoplasm, might perturb DNA replication. To test this, HSF8 cells were infected with GFP-HsCdc6 retroviruses. One day after infection, at a time when infected cells had only begun to express very low levels of GFP-HsCdc6wt, GFP-HsCdc6A1A2A3, GFP-HsCdc6E1E2E3, and GFP alone, the cells were synchronized in G_0 by serum deprivation for 3 days and then stimulated with serum in the presence of BrdUrd. At different times after serum addition, cells were fixed and stained with an anti-BrdUrd antibody to identify cells that had entered S phase. GFP-HsCdc6wt was localized either in the nuclei of cells without the BrdUrd staining or in the cytoplasm of cells with the BrdUrd staining, indicating that cytoplasmic relocalization of GFP-HsCdc6wt was tightly associated with initiation of DNA replication (Fig. 5*A*). The subcellular localization GFP-HsCdc6E1E2E3 was exclusively cytoplasmic, and GFP-HsCdc6A1A2A3 was exclusively nuclear regardless of

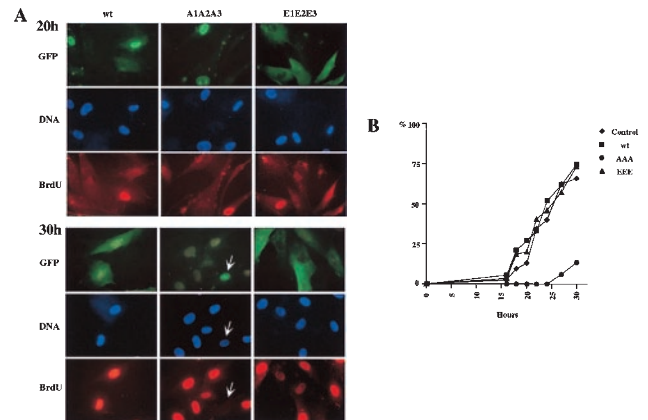

FIG. 5. Expression of the HsCdc6A1A2A3 mutant inhibits initiation of DNA replication. HSF8 cells expressing the indicated GFP-HsCdc6 proteins and uninfected control cells were synchronized in G_0 by serum deprivation and then stimulated with 20% FCS in fresh medium in the presence of 10 μM BrdUrd. Cells were fixed and stained with anti-BrdUrd antibody and Hoechst 33258 at the indicated times. (*A*) Representative photomicrographs of *B* at 20 and 30 h are shown. (*B*) Percentages of BrdUrd-positive cells in indicated points are shown. Each value represents a minimum count of 100 cells.

cell cycle stage. When the percentage of cells in S phase after serum stimulation was estimated (Fig. 5*B*), uninfected cells, HSF8 cells that expressed GFP (data not shown), GFP-HsCdc6wt, or GFP-HsCdc6E1E2E3, started to synthesize their DNA around 16 h after serum addition, and by 30 h, $\approx$70% of the cells had entered S phase. In contrast, HSF8 cells that expressed GFP-HsCdc6A1A2A3 had not started DNA synthesis even 24 h after serum addition, and, by 30 h, only a few cells, which had much weaker GFP signals, had entered S phase. We also observed that overexpression of HsCdc6A1A2A3 inhibited BrdUrd incorporation in asynchronously growing HSF8 cells (data not shown). These results indicated that ectopic expression of unphosphorylated HsCdc6 mutant, HsCdc6A1A2A3, inhibited initiation of DNA replication in HSF8 cells. The simplest interpretation of the inhibitory effect of overexpression of HsCdc6A1A2A3 (the level of expression of HsCdc6A1A2A3 was estimated to be $\approx$5-fold higher than that of endogenous HsCdc6) is that phosphorylation of HsCdc6 by Cdks is required for initiation of DNA replication. However, we cannot unequivocally rule out the possibility that HsCdc6A1A2A3 acts to sequester some component essential for initiation of DNA replication, whereas overexpressed HsCdc6wt does not. Further work with *in vitro* replication systems is required to establish whether Cdk-mediated phosphorylation of HsCdc6 is required for the initiation of DNA replication.

In summary, we have examined how HsCdc6 protein is regulated during the cell cycle and its functional role in controlling initiation of DNA replication in human cells. Unlike Cdc6p in yeast, the level of HsCdc6 protein is not regulated during the cell cycle in human cells. However, HsCdc6 is phosphorylated in a cell cycle-regulated fashion at sites phosphorylated by Cdks *in vitro*. We provide direct evidence that phosphorylation of HsCdc6 by Cdks is required for initiation of DNA replication and prevents its reassociation with chromatin via Crm1-dependent export from the nucleus. Based on our findings, together with the current model for DNA replication (1, 2), we present the following model for regulation of DNA replication by Cdk phosphorylation of HsCdc6 in mammalian cells (Fig. 6). We propose that phosphorylation of HsCdc6 by Cdks regulates DNA replication at at least two steps: first, by promoting initiation of DNA replication and, second, by preventing DNA rereplication. In G_1, when Cdk (mainly Cdk2) activity is at a minimum, HsCdc6

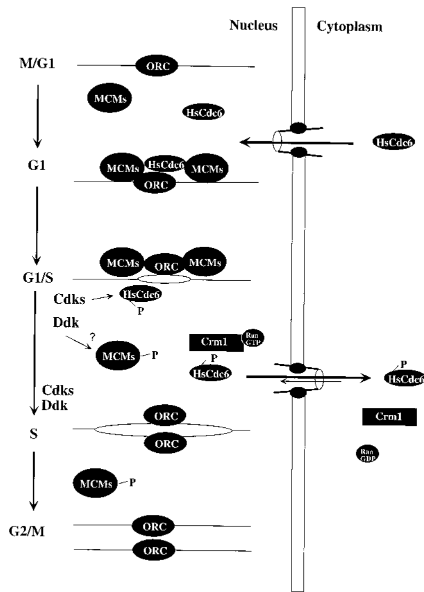

FIG. 6. Model for regulation of DNA replication by Cdk phosphorylation of HsCdc6 in human cells (for details, see text).

is not phosphorylated and is localized in the nucleus. Nuclear localization of HsCdc6 allows it to associate with the chromatin/nuclear matrix, presumably with ORC at replication origins. Association of HsCdc6 with ORC recruits other initiation factors, such as Mcm proteins, to establish pre-RCs at replication origins. At the G_1/S transition, activation of Cdk2 by association of cyclin E and cyclin A results in phosphorylation of HsCdc6 at the replication origins. Phosphorylation of HsCdc6 by Cdk2, possibly in conjunction with phosphorylation of other replication-initiation factors such as Cdc45 (40), is required for initiation of DNA replication. Exactly why phosphorylation of HsCdc6 is needed is unclear, but it could promote dissociation of HsCdc6 from pre-RCs, thus freeing Mcm proteins. Upon initiating DNA replication, phosphorylated HsCdc6 is transported from the nucleus to the cytoplasm by Crm1-dependent protein export. The absence of nuclear HsCdc6 during S, G_2, and M phase of the cell cycle prevents reassembly of pre-RCs on the fired origins, thereby preventing DNA rereplication. In this regard, HsCdc6 has properties akin to "licensing" factor, a role that previously has been suggested for Cdc6 (41). These dual consequences of phosphorylation of HsCdc6 by Cdks ensure that cells duplicate their DNA once and only once per cell cycle.

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1. Stillman, B. (1996) *Science* **274,** 1659–1664.
2. Dutta, A. & Bell, S. P. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 293–332.
3. Piatti, S., Lengauer, C. & Nasmyth, K. (1995) *EMBO J.* **14,** 3788–3799.
4. Detweiler, C. S. & Li, J. J. (1997) *J. Cell Sci.* **110,** 753–763.
5. Donovan, S., Harwood, J., Drury, L. S. & Diffley, J. F. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5611–5616.
6. Tanaka, T., Knapp, D. & Nasmyth, K. (1997) *Cell* **90,** 649–660.
7. Perkins, G. & Diffley, J. F. (1998) *Mol. Cell* **2,** 23–32.
8. Weinreich, M., Liang, C. & Stillman, B. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 441–446.
9. Elsasser, S., Lou, F., Wang, B., Campbell, J. L. & Jong, A. (1996) *Mol. Biol. Cell* **7,** 1723–1735.
10. Brown, G. W., Jallepalli, P. V., Huneycutt, B. J. & Kelly, T. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6142–6147.
11. Drury, L. S., Perkins, G. & Diffley, J. F. (1997) *EMBO J.* **16,** 5966–5976.
12. Kominami, K. & Toda, T. (1997) *Genes Dev.* **11,** 1548–1560.
13. Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D. & Kelly, T. J. (1997) *Genes Dev.* **11,** 2767–2779.
14. Liang, C. & Stillman, B. (1997) *Genes Dev.* **11,** 3375–3386.
15. Lopez-Girona, A., Mondesert, O., Leatherwood, J. & Russell, P. (1998) *Mol. Biol. Cell* **9,** 63–73.
16. Aladjem, M. I., Rodewald, L. W., Kolman, J. L. & Wahl, G. M. (1998) *Science* **281,** 1005–1009.
17. Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H. & Nojima, H. (1995) *Cell* **81,** 601–609.
18. Coleman, T. R., Carpenter, P. B. & Dunphy, W. G. (1996) *Cell* **87,** 53–63.
19. Romanowski, P., Madine, M. A. & Laskey, R. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10189–10194.
20. Rowles, A. & Blow, J. J. (1997) *Curr. Opin. Genet. Dev.* **7,** 152–157.
21. Hua, X. H. & Newport, J. (1998) *J. Cell Biol.* **140,** 271–281.
22. Williams, R. S., Shohet, R. V. & Stillman, B. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 142–147.
23. Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z. H., Hendricks, M., Parvin, J. D. & Dutta, A. (1998) *Mol. Cell. Biol.* **18,** 2758–2767.
24. Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R. & Williams, R. S. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3603–3608.
25. Stoeber, K., Mills, A. D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R. A. & Williams, G. H. (1998) *EMBO J.* **17,** 7219–7229.
26. Fukunaga, R. & Hunter, T. (1997) *EMBO J.* **16,** 1921–1933.
27. Jiang, W. & Hunter, T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14320–14325.
28. Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M. & Weinstein, I. B. (1993) *Oncogene* **8,** 3447–3457.
29. Jiang, W., Jimenez, G., Wells, N. J., Hope, T. J., Wahl, G. M., Hunter, T. & Fukunaga, R. (1998) *Mol. Cell* **2,** 877–885.
30. Boyle, W. J., van der Geer, P. & Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149.
31. Fujita, M., Kiyono, T., Hayashi, Y. & Ishibashi, M. (1997) *J. Biol. Chem.* **272,** 10928–10935.
32. Kimura, H., Takizawa, N., Nozaki, N. & Sugimoto, K. (1995) *Nucleic Acids Res.* **23,** 2097–2104.
33. Todorov, I. T., Attaran, A. & Kearsey, S. E. (1995) *J. Cell Biol.* **129,** 1433–1445.
34. Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J. & Helin, K. (1999) *EMBO J.* **18,** 396–410.
35. Stade, K., Ford, C. S., Guthrie, C. & Weis, K. (1997) *Cell* **90,** 1041–1050.
36. Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I. W. (1997) *Cell* **90,** 1051–1060.
37. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. & Nishida, E. (1997) *Nature (London)* **390,** 308–311.
38. Ullman, K. S., Powers, M. A. & Forbes, D. J. (1997) *Cell* **90,** 967–970.
39. Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S. & O'Shea, E. K. (1998) *Nature (London)* **396,** 482–486.
40. Zou, L. & Stillman, B. (1998) *Science* **280,** 593–596.
41. Botchan, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9997–10000.
42. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
43. Li, N. & Karin, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 13012–13017.