Replication initiation must be a carefully regulated process to avoid genomic instability caused by aberrant replication. In eukaryotic cells, distinct steps of protein loading (origin licensing) and replication activation are choreographed such that a cell can replicate only once per cell cycle. The first proteins recruited to the origins form the pre-replication complex. Of these proteins, Cdt1 is of interest, as it is the focus of several pathways to control replication initiation. It is degraded by two different pathways, mediated by the interaction of Cdt1 with proliferating cell nuclear antigen (PCNA) or with cyclin-Cdk2 and inhibited by geminin once cells are in S-phase, presumably to prevent reloading of pre-replication complexes once S-phase has begun. Although the requirement of Cdt1 in loading MCM2–7 is known, the mechanism by which overexpressed Cdt1 stimulates re-replication is unclear. In this study we have designed various mutations in Cdt1 to determine which portion of Cdt1 is important for re-replication, providing insight into possible mechanisms. Surprisingly, we found that mutants of Cdt1 that do not interact with MCM2–7 are able to induce re-replication when overexpressed. The re-replication is not due to titration of geminin from endogenous Cdt1 and is not accompanied by stabilization of endogenous Cdt1. Additionally, the N-terminal one-third of Cdt1 is sufficient to induce re-replication when overexpressed. The re-replication system of replication licensing relies on the sequential recruitment of replication initiation factors to chromatin at the appropriate time and prevents association at all other times. By controlling the chromatin association of these initiation factors, cells are able to limit precisely when replication can begin and prevent it otherwise.

In higher eukaryotes, it is generally thought that the origin recognition complex recognizes origins of replication. The origin recognition complex serves as an origin marker and recruits Cdc6 and Cdt1 to chromatin. These proteins are then required to load the MCM2–7 complex. Once the MCM2–7 complex is loaded, downstream members of the preinitiation complex, including Cdc45/Sld3, GINS, MCM10, and Dpb11/Sld2, are loaded onto chromatin, eventually culminating in recruitment of the replication machinery. The recruitment of these factors is controlled by the presence or absence of cyclin-dependent kinase (CDK) activity. CDK activity is low in G1, when the pre-replication complex (consisting of the origin recognition complex, Cdc6, Cdt1, and MCM2–7) is loaded. Increasing CDK activity is then required for further loading of the preinitiation complex members and the replication machinery (for review see Refs. 1 and 2).

MCM2–7 is thought to be the replicative helicase responsible for unwinding DNA ahead of the replication machinery (3–6). It is loaded in a Cdc6- and Cdt1-dependent manner, and this loading can only occur during low levels of CDK activity, in late M phase and early G1 phase. Cdt1 has been shown to associate physically with MCM2–7 in many organisms (7–10), suggesting that it directly recruits MCM2–7 to chromatin. However, Cdc6 ATPase mutants increase association of Cdt1 and decrease association of MCM2–7 with chromatin, which might suggest mutual exclusion of Cdt1 and MCM2–7 on chromatin (7, 11). Either way, it has been suggested that the interaction between Cdt1 and MCM2–7 is required to load the helicase on chromatin. Recent experiments in *Xenopus* egg extracts show that the C-terminal region of Cdt1 is required for MCM2–7 loading and that this region is also required for the interaction with MCMs (mini-chromosome maintenance) (10).
HsCdt1 Induces Re-replication Without MCM Interaction

importance of the C-terminal one-third of Cdt1 is highlighted by the fact that this is the only area that shows sequence identity from <i>Saccharomyces cerevisiae</i> to humans (7, 12). Once MCM2–7 is loaded, its subsequent activation (and eventual origin firing) requires CDK and CDK-like activity (for review see Ref. 2). Therefore, MCM2–7 bridges the two CDK activity states that function to license replication: its chromatin loading must occur in low CDK activity, and its activation and origin firing must occur in high CDK activity.

One of the key players in the replication licensing system is Cdt1. Cdt1 is required for MCM2–7 loading (7, 13, 14). MCM2–7 loading is limited to late M and early G1 phases in part due to inhibition of Cdt1 by several mechanisms in S-phase. Cdt1 is degraded in S-phase by two different mechanisms: CDK-dependent degradation by the SCF (Skp1/Cullin/S-Phase) F-box containing) complex and PCNA-dependent degradation by the Cul4-DDB1 complex (for review see Ref. 15). It is also inhibited by geminin (16, 17), a small protein that occurs at high levels during S-phase. Disrupting these mechanisms of Cdt1 inhibition can cause re-replication in a variety of systems (18–22). This suggests that when Cdt1 is not inhibited it can stimulate re-replication. Despite the expectation that inappropriate Cdt1 activity in S-phase may cause re-replication by excessive MCM2–7 reloading, the actual role of Cdt1 in re-replication induction is unclear.

Recent work has shown that Cdt1 levels are increased in certain cancers (23). Interestingly, in 73% of cases in which p53 was also mutated, aneuploidy was observed. Our laboratory has reported similar observations; in tumor cell lines lacking p53 and overexpressing Cdt1 and Cdc6, re-replication is observed (18). Furthermore, high levels of Cdt1 appear to have a causative role in cancer. It has been shown that retrovirally activated Cdt1 can be oncogenic in mice (24) and that Cdt1 overexpression in thymocytes can cause lymphomas when p53 is also mutated. As Cdt1 overexpression causes re-replication in a variety of systems, it is possible that high levels of Cdt1 induce genomic instability and tumorigenesis by inducing re-replication. Therefore, an understanding of the mechanism of re-replication initiation is also informative for tumorigenesis and disease progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T cells, human embryonic kidney cells transfected with adenovirus oncogenes E1a and E1b and with simian virus 40 oncogene T antigen, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% iron-supplemented donor calf serum and 1% penicillin/streptomycin. Standard tissue culture growth conditions and methods were used. Lipofectamine 2000 (Invitrogen) was used to transfect cells.

**Cdt1 Mutant Construction**—Cdt1 mutants were subcloned using PCR-based techniques in a vector with an N-terminal FLAG tag. Primer sequences used are available upon request. Because the natural nuclear localization sequence of Cdt1 is in the first 93 residues, the constructs lacking these N-terminal residues (94–X, 163–X) have an artificial nuclear localization sequence fused to the N terminus to ensure normal localization.

Western Blotting. Immunoprecipitation, and siRNA—Western blotting was performed as described. In this case, cells were lysed in 50 mM Tris, pH 7.4, 0.2% Nonidet P-40, 150 or 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and a 1:100 protease inhibitor mixture (Sigma). Equal amounts of protein were loaded on SDS-PAGE followed by transfer to nitrocellulose for standard Western blotting. FLAG and β-actin antibodies were purchased from Sigma, and MCM6 and cyclin A antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG agarose beads (Sigma) were used for Cdt1 co-immunoprecipitation. Geminin antibody was described previously (16). hsCdt1 antibody was described previously (25). siRNA was performed according to common methods using RNAi MAX (Invitrogen). siRNA oligonucleotides against human geminin have been described previously (20). The Cdt1 siRNA sequence is 5’-GCAUGUUUGCAGAUA-3’.

FACS—Cells were prepared as described previously (18). Cells were cotransfected with farnesylated EGFP and gated for GFP-positive cells to ensure analysis was limited to the Cdt1-transfected cells. Alternately, cells were transiently transfected with a puromycin resistance marker, and 48 h of puromycin treatment was used to enrich transfected cells. The analysis was carried out on a BD Biosciences FACS Calibur using Cellquest and Flojo software.

**Chromatin Fractionation**—Chromatin fractionation protocols used to observe MCM7 and Cdt1 chromatin loading were described previously (Refs. 26 and 27, respectively). Briefly, the MCM7 chromatin loading was observed as follows. Cells (2 × 10⁶) were lysed in 100 μl of CSK buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) containing 0.5% Triton X-100, 1 mM ATP, and 1 mM Na3VO4. Lysates were incubated on ice for 20 min and then centrifuged at 1500 rpm for 5 min at 4 °C. Supernatant (S1) was removed, and pellets were washed with 1 ml of lysis buffer and centrifuged again. Pellets were incubated in 100 μl of lysis buffer containing 1 mM CaCl2 and 120 units of micrococcal nuclease (Worthington) for 10 min at 37 °C and centrifuged. Supernatant (S2, chromatin-bound fraction) was removed, and pellets were washed with 1 ml of lysis buffer and centrifuged again. Pellets were boiled in 100 μl of 1× sample buffer (P2) (26).

The Cdt1 chromatin loading was determined as follows. Cells were resuspended (4 × 10⁶ cells/ml) in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, a 1:100 protease inhibitor mix (Sigma), 0.1 mM phenylmethylsulfonyl fluoride). 0.1% Triton X-100 was added, and the cells were incubated for 5 min on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation (4 min, 1,300 × g, 4 °C). The supernatant (S1) further clarified by high-speed centrifugation (15 min, 20,000 × g, 4 °C) to remove cell debris and insoluble aggregates. Nuclei were washed once in Buffer A and then lysed in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (4 min, 1,700 × g, 4 °C), washed once in Buffer B, and centrifuged again under the same conditions. The final chromatin pellet (P3) was resuspended in Laemmli buffer and sheared by passing it through a syringe. To release chromatin-
bound proteins by nuclease treatment, cell nuclei (P1) were resuspended in Buffer A plus 1 mM CaCl₂ and 0.2 unit of micrococcal nuclease (Sigma). After incubation at 37 °C for 2 min, the nuclease reaction was stopped by the addition of 1 mM EGTA. Nuclei were collected by low-speed centrifugation and lysed as above (27).

**RESULTS**

Recent evidence has suggested that the C terminus of *Xenopus* and mouse Cdt1 interacts with the MCM2–7 complex. We first sought to confirm this result in human cells. A variety of truncations and internal deletions were made in the regions defined in mouse (9) and *Xenopus* (10) systems to be important for the interaction with MCM2–7. To determine the analogous amino acids in hsCdt1 (*Homo sapiens*) (546 amino acids), a multiple alignment was performed using ClustalW (28). In *Xenopus* egg extracts, xlCdt1/H9004 573–620 (*X. laevis*) did not bind MCM2–7. The analogous hsCdt1 residues (499–546) were therefore deleted. Yeast two-hybrid and in vitro pulldown experiments with mouse Cdt1 suggested that mmCdt1 (407–477) (*Mus musculus*) was important for direct interaction with mmMCM6. Interestingly, mmCdt1 Δ520–577 (a deletion of the extreme C terminus) seemed to interact more strongly with mmMCM6, suggesting that this region may be inhibitory for MCM binding. We synthesized the analogous deletions of the internal required region and C-terminal inhibitory region in human Cdt1: hsCdt1 Δ395–465 and hsCdt1 Δ508–546. Additional internal deletions were also made based on a secondary structure prediction using the GOR4 algorithm (29). The fourth-from-last helix (hsCdt1 Δ444–465), the third-from-last helix (hsCdt1 Δ495–505), and the final helix (hsCdt1 Δ518–546) were each deleted. These mutations are summarized in Fig. 1.

To test the interaction of these hsCdt1 deletions with MCM2–7, we overexpressed FLAG-tagged versions of the proteins in human 293T cells. hsCdt1 was then immunoprecipitated using an anti-FLAG antibody, and hsMCM6 levels were examined by Western blotting. Wild type hsCdt1 was able to pull down endogenous hsMCM6, reproducing earlier results in different systems from other laboratories (Fig. 2A). However, none of the deletion mutants pulled down detectable hsMCM6, indicating that a large portion of the C terminus of hsCdt1 is important for the interaction with hsMCM6. Both the wild type and deletion mutants of hsCdt1 were able to pull down geminin, indicating the proteins are likely to be folded correctly. Interestingly, both Δ499–546 and Δ508–546 did not interact with hsMCM6. In previous studies, the Δ499–546 *Xenopus* equivalent did not bind to MCM2–7 (10), but the mouse Δ508–546 equivalent bound more strongly (9), suggesting that the extreme C terminus of Cdt1 was inhibitory for MCM binding. Our data agrees with the *Xenopus* data, as the extreme C terminus appears to be required for MCM interaction. It is possible that the Cdt1–MCM6 interaction in mice is slightly different from that in other organisms.

To eliminate the possibility that these deleted regions are also important for a non-MCM binding function of hsCdt1, we
made three different sets of point mutants in full-length hsCdt1. These mutants are termed PEHL (P492T, E496A, H498A, L502A), RELAR (R453A, E456A, L457A, A458S, R459A), and RPLVF (R356K, P455H, L461E, V464A, F465A). Each set of point mutants was made in highly conserved residues in the regions deleted previously (see Fig. 1). FLAG-tagged versions of these point mutants were expressed in 293T cells and pulled down with anti-FLAG. Although wild type hsCdt1 interacted with hsMCM6, we did not observe an interaction between any of our point mutants and hsMCM6 (Fig. 2B), suggesting that these mutations interrupt the binding between the two proteins. Again, the hsCdt1 point mutants all bind to geminin. As only 4–5 amino acids have been changed in each mutant, the chance of disrupting another activity of Cdt1 in all three cases is decreased.

We tested the re-replication initiation activity of these point mutants by overexpressing them in human 293T cells. Our laboratory has shown previously that by either overexpressing hsCdt1 (18) or relieving its inhibition via knockdown of geminin (20, 30), re-replication can be induced in human cells. As expected, transient overexpression of wild type full-length hsCdt1 induced >4N DNA content as determined by FACS (Fig. 3A). In addition, overexpression of each of the MCM non-interacting point mutants also induces re-replication (Fig. 3A). The percentage of re-replicating cells after hsCdt1 point mutant overexpression is similar or higher than wild type hsCdt1 (quantitated in Fig. 3B). This result is very surprising, as these point mutants of hsCdt1 do not appear to interact with hsMCM6. This suggests that although Cdt1 is required for MCM2–7 loading onto chromatin, the interaction between these proteins is not required for re-replication induction.

Because the MCM non-interacting point mutants of Cdt1 were able to induce re-replication, we examined the ability of C-terminal deletion mutants to do the same. In previous Xenopus experiments, a C-terminal truncation neither interacted with MCM2–7 nor functioned in replication licensing. The analogous human mutant, residues 1–498, as well as two mutants with larger C-terminal deletions, residues 1–389 and

---

**FIGURE 3.** Overexpression of wild type and MCM non-interacting mutants of hsCdt1 cause re-replication. A, DNA content after overexpression of indicated hsCdt1 mutants (and farnesylated EGFP as a transfection marker) was determined by propidium iodide FACS. Transfected cells were selected for analysis based on positive GFP expression. Cells with >4N DNA content (re-replicating cells) are identified by a bracket, and the percentage of total cells is shown. B, quantitation of percent of cells with >4N DNA content (re-replication) is shown. C, quantitation of percent re-replication observed after overexpression of wild type Cdt1 and C-terminal truncations is shown. D, a Western blot showing the expression of Cdt1 and deletion mutants is shown.
HsCdt1 Induces Re-replication Without MCM Interaction

1–370, was tested for its ability to induce re-replication. Again, surprisingly, these MCM non-interacting mutants caused re-replication (Fig. 3, C and D). This further supports the notion that direct interaction between the overexpressed Cdt1 and MCM2–7 is not required for re-replication. Additionally, these results indicate that the entire C-terminal region, although the only part conserved from S. cerevisiae to humans, is not required to induce re-replication, suggesting that re-replication may not depend on the normal licensing role of Cdt1.

One possible explanation for the induction of re-replication by MCM non-interacting point mutants of Cdt1 is that the overexpressed Cdt1 is simply titrating geminin away from endogenous Cdt1, thereby activating it. We addressed this possibility by overexpressing the central domain of Cdt1 that contains a geminin-interacting region: hsCdt1-(163–370). This FLAG-tagged fragment is able to pull down equal amounts of geminin compared with wild type and point mutant hsCdt1, indicating it is able to titrate geminin just as efficiently (Fig. 4A). However, this fragment is not able to induce re-replication compared with wild type hsCdt1 (Fig. 4B); to ensure proper localization, this fragment and others with large N-terminal deletions have an artificial nuclear localization sequence added to the N terminus. This leads us to believe that the re-replication stimulation observed after overexpression of hsCdt1 is not due to geminin titration.

To further demonstrate that Cdt1 overexpression does not simply titrate geminin away from endogenous Cdt1, siRNA was used to knock down geminin followed by Cdt1 overexpression. Although re-replication is induced after geminin siRNA in some cell lines (20, 31), we did not observe significant re-replication after geminin siRNA in 293T cells (Fig. 4, C and D, FLAG lanes). Taken on its own, this observation suggests that geminin depletion from endogenous Cdt1 is not sufficient to induce re-replication in this cell line. Wild type Cdt1 was also overexpressed in control or geminin siRNA-treated cells. Geminin depletion in Cdt1-overexpressing cells had very little effect on the percentage of re-replicating cells. (Fig. 4, C and D) Thus, Cdt1 overexpression induces re-replication by a mechanism other than titration of geminin.

It is possible that overexpressed Cdt1 may titrate another inhibitor of re-replication. CDK activity is also important for inhibition of re-replication as has been demonstrated recently (30). As Cdt1 contains a cyclin (Cy) binding motif that is required for interaction with cyclins, titration of cyclins from endogenous targets (including endogenous Cdt1) could cause re-replication. To test this, we deleted the Cy motif in both full-length Cdt1 and Cdt1-(1–370) (Fig. 4, E and F). Although the ΔCy mutants do not interact with cyclin A (Fig. 4G) (32), they are still able to induce re-replication, suggesting that titration of cyclins is not required for the observed re-replication following Cdt1 overexpression.

It is also interesting to note that although the 163–370 fragment of Cdt1 did not induce re-replication, the 1–370 fragment did. This indicates that a critical function of the N terminus of Cdt1 is required for re-replication. To further examine this phenomenon, we overexpressed the 163–546 fragment, in which only the N terminus is deleted. Surprisingly, we found that this fragment induced an S-phase arrest (Fig. 5, A and B). A slightly shorter N-terminal truncation, fragment 94–546, also induced an S-phase arrest. Interestingly, if we disrupted MCM binding by overexpressing a Cdt1 mutant lacking the N terminus and lacking the Cy motif (ΔCy) of Cdt1 is shown. E, percent re-replication observed after overexpression of wild type and mutants lacking the cyclin-interacting Cy motif (ΔCy) of Cdt1 is shown. F, Western blots show the expression of Cdt1 mutants used in E, G, a Western blot shows cyclin A protein co-immunoprecipitating with FLAG-Cdt1 but not with the ΔCy mutant.

FIGURE 4. Re-replication stimulation by different domains of Cdt1. A, a Western blot shows FLAG immunoprecipitation (FLAG-IP) as in Fig. 2. Arrow mark the position of immunoprecipitated FLAG-Cdt1. Note that Cdt1-(163–370) binds geminin. B, quantitation of percent re-replication observed after overexpression of hsCdt1 wild type and fragment 163–370 is shown. Note that the 163–370 fragment does not induce re-replication. C, Western blots indicating geminin knockdown after siRNA treatment and FLAG-Cdt1 overexpression are shown. GL2, control oligo targeting luciferase. D, quantitation of percent re-replication observed after depletion of geminin and overexpression of Cdt1 is shown. E, percent re-replication observed after overexpression of wild type and mutants lacking the cyclin-interacting Cy motif (ΔCy) of Cdt1 is shown. F, Western blots show the expression of Cdt1 mutants used in E. G, a Western blot shows cyclin A protein co-immunoprecipitating with FLAG-1–370 but not with the ΔCy mutant.
HsCdt1 Induces Re-replication Without MCM Interaction

FIGURE 5. The role of the N terminus in re-replication induction. A, the FACS profile of DNA content after expression of different Cdt1 fragments that lack the N terminus. Note the mid-S-phase peak that is present when the N terminus is deleted; it depends on the MCM-interacting domains. B, expression levels of overexpressed Cdt1 and truncation mutants by anti-FLAG Western blot. C, quantitation of percent re-replication after expression of the N-terminal one-third of hsCdt1 and Cdt1 lacking the first five amino acids (important for PCNA-mediated degradation). D, anti-FLAG Western blot showing expression of Cdt1 mutants used in C.

Earlier we observed that titration of PCNA or cyclin A by exogenous Cdt1 was not required for re-replication; preventing the titration of either cyclin A or PCNA did not disrupt re-replication induction. But what if titration of either one of these binding protein, shifts from P3 to S3 with MNase treatment. Interestingly, a fraction of the overexpressed Cdt1 deletion mutants are present in the chromatin-bound protein fraction just as Orc2 (Fig. 6A).

Because these mutant Cdt1 proteins stimulate re-replication and can be found on chromatin, we wondered whether they stimulated MCM loading on chromatin. A more stringent chromatin fractionation protocol was used to better visualize MCM chromatin loading. In this method, the S1 fraction contains proteins solubilized by medium-salt, medium-detergent buffer. P1 is then treated with MNase, releasing chromatin-bound proteins into the soluble S2 fraction. Proteins not solubilized by MNase treatment remain in the insoluble P2 fraction. We examined the MCM7 levels in the S2 fraction and found that the levels appear to be equal after Cdt1-(1–370) overexpression (Fig. 6B). However, MCM is released from chromatin as cells pass through S-phase, so a confounding factor is that more of the re-replicating cells are in S-phase and post-S-phase. Indeed, after Cdt1-(1–370) overexpression the percentage of G1 cells is decreased to about half that of control cells (Fig. 6C), and chromatin-bound MCM levels are expected to be low. Nevertheless, the levels of chromatin-bound MCM are equal in the two populations, implying that chromatin loading of MCM is increased by the exogenous overexpressed Cdt1 mutant.

It is therefore possible that the exogenous Cdt1 that cannot interact with MCM is overactivating endogenous Cdt1 to load cellular MCM onto chromatin. We tested this by knocking down endogenous Cdt1 using siRNA in cells overexpressing FLAG-Cdt1 (residues 1–498), which does not interact with MCM. The overexpressed exogenous FLAG-Cdt1 was not detectably decreased by the siRNA, whereas the endogenous Cdt1 was decreased (Fig. 6E). Intriguingly, selective decrease of the endogenous Cdt1 decreases the Cdt1 (residues 1–498)-stimulated re-replication (Fig. 6D). This indicates that overexpressed Cdt1 requires the presence of endogenous Cdt1 to induce re-replication and may induce re-replication by overactivating endogenous Cdt1 to load cellular MCM.

To understand how the C-terminally deleted Cdt1 that does not interact with MCM induces re-replication, we examined whether it was loaded on chromatin (Fig. 6A). The S1 fraction represents the soluble fraction after low-salt, mild-detergent lysis (data not shown). It is clarified by a high-speed spin to yield S2. After hypotonic buffer treatment of P1 (thought to be nuclei), soluble fraction S3 and pellet P3 are recovered. Proteins bound to chromatin would be released by micrococcal nuclease (MNase) from P3 to the S3 fraction. Orc2, a control chromatin-
proteins, PCNA or cyclin A, alone were sufficient to induce re-replication? If this were true, then overexpressing a Cdt1 fragment mutated for both PCNA and cyclin interaction would not be able to stimulate re-replication. This possibility was tested by overexpressing the double mutant Cdt1-(6–370)/H9004Cy.

Although the Cdt1-(6–370) mutant induced re-replication, Cdt1-(6–370)/H9004Cy did not, indicating that titrating at least one of the two Cdt1-interacting proteins is required to induce re-replication (Fig. 7, A and B).

Titrating the destruction mechanisms seems to overactivate Cdt1. As both pathways degrade Cdt1, the inhibition of these pathways may stabilize endogenous Cdt1. This was tested by examining the endogenous Cdt1 levels after overexpressing Cdt1-(1–370) and decrease of endogenous Cdt1 by siRNA. E, Western blot showing endogenous Cdt1 is decreased after siRNA, but exogenous Cdt1 is equal. Endogenous Cdt1 is detected in the control empty FLAG-vector transfection (first and second lanes), as the exogenous Cdt1 obscures it in the FLAG-Cdt1 (residues 1–498) transfection (third and fourth lanes). Both endogenous and exogenous Cdt1 are detected with anti-Cdt1 antibody.

proteins, PCNA or cyclin A, alone were sufficient to induce re-replication? If this were true, then overexpressing a Cdt1 fragment mutated for both PCNA and cyclin interaction would not be able to stimulate re-replication. This possibility was tested by overexpressing the double mutant Cdt1-(6–370)/ΔCy. Although the Cdt1-(6–370) mutant induced re-replication, Cdt1-(6–370)/ΔCy did not, indicating that titrating at least one of the two Cdt1-interacting proteins is required to induce re-replication (Fig. 7, A and B).

Titrating the destruction mechanisms seems to overactivate Cdt1. As both pathways degrade Cdt1, the inhibition of these pathways may stabilize endogenous Cdt1. This was tested by examining the endogenous Cdt1 levels after overexpressing Cdt1-(1–370), which induces re-replication. We transiently transfected cells with a Cdt1-overexpressing plasmid and a puromycin resistance plasmid, and 24 h after transfection we subjected them to puromycin selection for 48 h to enrich the Cdt1-transfected cells. 48 h of puromycin selection is enough to kill untransfected cells, and indeed we could see robust re-replication when all surviving cells were examined at this stage (Fig. 7D). However, endogenous Cdt1 levels were not increased after overexpression of Cdt1 (Fig. 7C), indicating that endogenous Cdt1 may be overactivated without an obvious increase in the level of total Cdt1.

**DISCUSSION**

Genomic stability is of paramount importance to a cycling cell. To protect genomic integrity, cells have evolved a complex regulatory mechanism to ensure the genome is copied exactly once per cell cycle. Cdt1 regulation is an important aspect of this mechanism. Its destruction and inhibition in S-phase prevents re-replication, protecting the genome. Perturbing this inhibition by overexpressing Cdt1 or preventing its degradation or geminin-mediated inhibition causes re-replication. However, the way in which Cdt1 overexpression causes re-replication is not clear. In this study we have shown that mutations in Cdt1 that disrupt physical interaction with MCM2–7 are still able to function in licensing normal replication (10). This suggests that Cdt1 does not induce re-replication in human cells via its normal replication initiation function. Indeed, the N-terminal one-third of Cdt1 alone is able to stimulate re-replication, despite the fact that two-thirds of the protein (including the highly conserved C terminus) is missing. This result is rather puzzling and seems to contradict expectations that Cdt1 overexpression might cause re-replication by inappropriately recruiting MCM2–7 to chromatin via protein-protein interactions during S-phase.

If the stable, direct interaction between the C terminus of Cdt1 and MCM2–7 is not required for re-replication, how does exogenous Cdt1 function? One possibility is that the N-terminal part of exogenous Cdt1 is bridged to MCM2–7 via another protein and still functions to physically recruit MCM2–7 to chromatin despite the lack of the C-terminal interaction with MCM. It was reported recently that cyclin E protein, but not its
HsCdt1 Induces Re-replication Without MCM Interaction

If Cdt1 does not induce re-replication in a positive fashion through its normal replication initiation activity, perhaps it acts in a negative fashion by inhibiting an S-phase inhibitor. Cdt1 interacts well with (and is inhibited by) the S-phase inhibitor protein geminin. It is possible that exogenous Cdt1 titrates geminin away from endogenous Cdt1, overactivating it. However, expression of the central domain of Cdt1 did not induce re-replication, despite the observation that it was able to titrate geminin (Fig. 4, A and B). Therefore, titration of geminin alone is not sufficient to induce re-replication. Exogenous Cdt1 could also titrate the destruction machinery away from endogenous Cdt1, preventing its degradation. In fact, a Cdt1 mutant missing both the PCNA and cyclin A binding motifs is not able to induce re-replication (Fig. 7, A and B). Titrating only one of the two mechanisms is sufficient for exogenous Cdt1 to stimulate re-replication. This explains the observation that mutating one interaction motif did not disrupt re-replication mediated by overexpressed Cdt1; the other pathway (PCNA or cyclin A) was sufficient for re-replication. Only when titration of both PCNA and cyclin A was disrupted was re-replication prevented. This means that in this system, the inhibitory activity of both PCNA and cyclin A is required to prevent re-replication, suggesting that the two pathways are not redundant; they are both absolutely required for inhibiting endogenous Cdt1.

However, as Cdt1 is stabilized in S-phase only when both PCNA/Cul4 and cyclin A/Cul1-mediated pathways are inhibited (25), it seems surprising that titrating one alone could activate endogenous Cdt1. In fact, no increase in endogenous Cdt1 level is observed following overexpression of Cdt1-(1–370) (Fig. 7C). It is possible that titrating both degradation pathways stabilizes a subpopulation of endogenous Cdt1, thereby overactivating it. An increase in this special subpopulation will not be apparent when examining total cellular Cdt1 or even the chromatin-associated Cdt1 (data not shown). The other possibility is that Cdt1 is inhibited simply by the stable association with cyclin A and PCNA. The exogenous Cdt1 needs to titrate either of these proteins to disrupt inhibition by stable association, allowing the overactivation of endogenous Cdt1 without extensive stabilization. Although it is also possible that titration of the two destruction mechanisms stabilizes or activates another positive replication factor, endogenous Cdt1 is required for re-replication (Fig. 6D), so it is the likely target for overactivation. We imagine a model in which exogenous Cdt1 titrates PCNA and/or cyclin A away from endogenous Cdt1. This disrupts the normal inhibition of Cdt1 during S-phase, causing an inappropriate increase in MCM2–7 loading and resulting in re-replication (Fig. 7E).

Our results suggest that sustained interaction between overexpressed Cdt1 and MCM2–7 is detrimental to DNA replication if the degron in the N-terminal 94 residues of Cdt1 is deleted. We have shown previously that overexpression of non-degradable Cdt1 delays progression through S-phase in HeLa cells (36), and this result is reproduced in 293T cells, where Cdt1-(94–546) or Cdt1-(163–546) caused a pronounced S-phase accumulation. This accumulation was greatly diminished upon introduction of mutations that disrupt the stable association of MCM2–7 with Cdt1 (RPLVF or Δ499–C). Thus,
although Cdt1 is required to recruit MCM2–7 to origins in G1, and although S. cerevisiae Cdt1 (TAH11) is in a stable complex with MCM2–7 (7), persistent interaction between non-degradable Cdt1 and MCM2–7 in S-phase leads to problems in cell cycle progression.

The role of Cdt1 in re-replication is interesting and important. In higher eukaryotes, many pathways converge upon Cdt1 to prevent re-replication, including degradation in S-phase by two distinct pathways and inhibition by geminin. Cdt1 levels are often elevated in tumors (23), and overexpression of Cdt1 in mice can lead to genome instability in some cancers where there is not an overt increase in Cdt1 protein.

Acknowledgment—We thank members of the Dutta laboratory for critical discussion and suggestions.

REFERENCES

1. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
2. Teer, J. K., and Dutta, A. (2006) Results Probl. Cell Differ. 42, 31–63
3. Ishimi, Y. (1997) J. Biol. Chem. 272, 24508–24513
4. You, Z., Komamura, Y., and Ishimi, Y. (1999) Mol. Cell. Biol. 19, 8003–8015
5. Lee, J. K., and Hurwitz, J. (2000) J. Biol. Chem. 275, 18871–18878
6. Moyer, S. E., Lewis, P. W., and Botchan, M. R. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 10236–10241
7. Tanaka, S., and Diffley, J. F. (2002) Nat. Cell Biol. 4, 198–207
8. Cook, J. G., Park, C., Burke, T. W., Leone, G., Degregori, J., Engel, A., and Nevins, J. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1347–1352
9. Yanagi, K., Mizuno, T., You, Z., and Hanaoka, F. (2002) J. Biol. Chem. 277, 40871–40880
10. Ferenbach, A., Li, A., Brito-Martins, M., and Blow, J. J. (2005) Nucleic Acids Res. 33, 316–324
11. Randell, J. C., Bowers, J. L., Rodriguez, H. K., and Bell, S. P. (2006) Mol. Cell 21, 29–39
12. Devault, A., Vallen, E. A., Yuan, T., Green, S., Bensimon, A., and Schwob, E. (2002) Curr. Biol. 12, 689–694
13. Maiorano, D., Moreau, J., and Mechali, M. (2000) Nature 404, 622–625
14. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000) Nature 404, 625–628
15. Fujita, M. (2006) Cell Div. 1, 22
16. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) Science 290, 2309–2312
17. Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J. J. (2001) Nat. Cell Biol. 3, 107–113
18. Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003) Mol. Cell 11, 997–1008
19. Zhong, W., Feng, H., Santiago, F. E., and Kipreos, E. T. (2003) Nature 423, 885–889
20. Zhu, W., Chen, Y., and Dutta, A. (2004) Mol. Cell Biol. 24, 7140–7150
21. Arias, E. E., and Walter, J. C. (2005) Genes Dev. 19, 114–126
22. Li, A., and Blow, J. J. (2004) EMBO J. 23, 395–404
23. Karakoidos, P., Taraviras, S., Vassiliou, L. V., Zacharatos, P., Kastrinakis, N. G., Kougiou, D., Kouloukousa, M., Nishitani, H., Papavassiliou, A. G., Lygerou, Z., and Gorgoulis, V. G. (2004) Am. J. Pathol. 165, 1351–1365
24. Arentson, E., Faloone, P., Seo, J., Moon, E., Studts, J. M., Fremont, D. H., and Choi, K. (2002) Oncogene 21, 1150–1158
25. Senga, T., Sivaprasad, U., Zhu, W., Park, J. H., Arias, E. E., Walter, J. C., and Dutta, A. (2006) J. Biol. Chem. 281, 6246–6252
26. Todorov, I. T., Attaran, A., and Kearsey, S. E. (1995) J. Cell Biol. 129, 1433–1445
27. Mendez, J., and Stillman, B. (2000) Mol. Cell Biol. 20, 8602–8612
28. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
29. Garnier, J., Gibrat, J. F., and Robson, B. (1996) Methods Enzymol. 266, 540–553
30. Machida, Y. I., and Dutta, A. (2007) Genes Dev. 21, 184–194
31. Zhu, W., and Dutta, A. (2006) Mol. Cell 26, 4601–4611
32. Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004) J. Biol. Chem. 279, 19691–19697
33. Geng, Y., Lee, Y. M., Welcker, M., Swanger, J., Zagozdzon, A., Winer, J. D., Roberts, J. M., Kaldes, P., Clurman, B. E., and Sicinski, P. (2007) Mol. Cell 25, 127–139
34. Dominguez-Sola, D., Ying, C. Y., Grandori, C., Ruggiero, L., Chen, B., Li, M., Galloway, D. A., Gu, W., Gautier, J., and Dalla-Favera, R. (2007) Nature 448, 445–451
35. Cook, J. G., Chasse, D. A., and Nevins, J. R. (2004) J. Biol. Chem. 279, 9625–9633
36. Takeda, D. Y., Parvin, J. D., and Dutta, A. (2005) J. Biol. Chem. 280, 23416–23423

HsCdt1 Induces Re-replication Without MCM Interaction