DNA replication is controlled by the stepwise assembly of a pre-replicating complex and the replication apparatus. Cdt1 is a novel component of the pre-replicative complex and plays a role in loading the minichromosome maintenance (MCM) 2–7 complex onto chromatin. Cdt1 activity is inhibited by geminin, which is essential for the G2/M transition in metazoan cells. To understand the molecular basis of the Cdt1-geminin regulatory mechanism in mammalian cells, we cloned and expressed the mouse Cdt1 homologue cDNA in bacterial cells and purified mouse Cdt1 to near homogeneity. We found by yeast two-hybrid analysis that mouse Cdt1 associates with geminin, MCM6, and origin recognition complex 2. MCM6 interacts with the Cdt1 carboxyl-terminal region (amino acids 407–477), which is conserved among eukaryotes, whereas geminin associates with the Cdt1 central region (amino acids 177–380), which is conserved only in metazoans. In addition, we found that Cdt1 can bind DNA in a sequence-, strand-, and conformation-independent manner. The Cdt1 DNA binding domain overlaps with the geminin binding domain, and the binding of Cdt1 to DNA is inhibited by geminin. Taken together, we have defined structural domains and novel biochemical properties for mouse Cdt1 that suggest that Cdt1 behaves as an intrinsic DNA binding factor in the pre-replicative complex.

Chromosomal DNA replication is subject to strict cell cycle control, which ensures that cells enter S phase once and only once per cell cycle. A considerable body of evidence from both genetic analyses of yeast mutants and biochemical studies using Xenopus egg extracts has shown that the initiation of replication requires the stepwise assembly of protein complexes on chromatin to form a pre-replicative complex (pre-RC)1 (1–8). The pre-RC includes the origin recognition complex (ORC), the minichromosome maintenance protein complex (MCM), and the Cdc6 and Cdt1 proteins. After the activation of S phase-promoting kinases, CDKs, and the Dbf4-dependent kinase, DNA helicase unwinds the two DNA strands, and replication protein A stabilizes single-stranded DNA, thereby allowing an initiation complex to be formed by the loading of DNA polymers onto the pre-RC. Because most of the components of the pre-RC identified in Saccharomyces cerevisiae and Xenopus have been found in other eukaryotes including humans, it is believed that the mechanisms controlling the initiation of replication are conserved in all eukaryotes. However, the DNA helicase that is associated with the replication fork has not yet been identified, even in S. cerevisiae (1, 6, 8, 9). The best candidate for the replicative DNA helicase is the MCM2–7 complex. The MCM2–7 complex was first identified as a set of genes required for minichromosome maintenance in S. cerevisiae, and it was subsequently identified as a critical component of the replication licensing system in Xenopus egg extracts (3, 9, 10). MCM2–7 proteins are loaded onto chromatin at late telophase and gradually released as replication forks proceed, and concomitantly, chromatin undergoes a transition to the unlicensed state (3, 8, 11). The mouse and Schizosaccharomyces pombe MCM4/6/7 complexes possess low but significant DNA helicase activity (12–14). The presence of single-stranded tails in forked DNA substrates stimulates the processive helicase activity of the S. pombe MCM4/6/7 complex, suggesting that MCM may function as an intrinsic processive DNA helicase whose activity is controlled by as yet undefined modifications or uncharacterized interactions with other factors (15).

Thus, to understand the function of the MCM helicase in replication, it is important to determine not only the mechanism by which MCM is loaded onto chromatin but also the interplay between MCM and other replication components of the pre-RC at the onset of replication.

Two factors have been identified as necessary to load MCM proteins onto chromatin: Cdc6 and Cdt1 (1, 2, 4, 8, 16). Cdc6 is an essential component of the pre-RC and is conserved among organisms ranging from yeast to humans. Cdc6 is a member of a large superfamily of ATPases known as the AAA+ family, and it exhibits significant sequence similarity to subunits of clamp-loading proteins in eukaryotes (the replication factor C complex) and prokaryotes (the y-complex), which load the ring-shaped sliding clamp onto DNA (17). Interestingly, S. pombe, mouse, and archaeal MCM complexes have been observed by electron microscopic analysis to have a donut-like shape (18–20). The loading of MCM onto chromatin is dependent on ORC and Cdc6 in S. cerevisiae, S. pombe, Xenopus, and humans (1, 3, 4, 16).
9, 10). Therefore, Cdc6 has been considered to function as a clamp loader that facilitates the loading of the ring-shaped MCM–2–7 complex onto DNA. However, the in vitro subunit composition of the MCM complex remains unclear. In addition, the loading of the MCM–2–7 complex onto chromatin has not yet been reconstituted in vitro. Moreover, the stoichiometry of the pre-RC components, including the ORC, the MCM complex, and Cdc6, as well as the local architectural features that are conducive to pre-RC formation and the regulatory mechanisms that govern the conversion of the pre-RC to an initiation complex, remain to be clarified.

Recently, a novel component of the pre-RC, termed Cdt1, has been identified (2, 16). Cdt1 was first identified in S. pombe as a gene induced by the CDC1-dependent transcription factor cdc10 (21). In both S. pombe and Xenopus, Cdt1 has been shown to be essential for chromosomal replication and the assembly of MCM–2–7 on chromatin (22, 23). The association of Cdt1 with the pre-RC depends on ORC but is independent of Cdc6 and MCM2–7 on chromatin (22, 23). Cdt1 appears to be identical to the essential homologue of Cdt1 has also been isolated (26); the pre-RC components, including the ORC, the MCM complex, and Cdc6, as well as the local architectural features that are conducive to pre-RC formation and the regulatory mechanisms that govern the conversion of the pre-RC to an initiation complex, remain to be clarified.

Expression and Purification of Recombinant Proteins in E. coli—Expression and purification of recombinant proteins were carried out using the E. coli strain BL21 (DE3) and BL21(DE3) (NovaGen). Isopropyl-β-D-thiogalactoside (IPTG) was used to induce protein expression. The induced cells were harvested by centrifugation and washed in Buffer A (50 mmol/l Tris–HCl, pH 8, 1 mmol/l EDTA, and 25 g/l glycerol). The cells were lysed by sonication in Buffer A containing 1% Triton X-100. The supernatant was collected by centrifugation at 6,000 rpm for 20 min at 4°C. The supernatant was subjected to an initial purification on a protein G-agarose column. The target proteins were purified either by affinity chromatography using a protein G-agarose column or by size-exclusion chromatography on a Superdex-200 preparative column (Amersham Biosciences). Gel filtration purification steps were performed using a Superdex 200 16/60 column (Amersham Biosciences) at a flow rate of 1 ml/min. The target proteins were collected and concentrated by using Centriplus-10 concentrators (Millipore). The purity of the purified proteins was verified by SDS-PAGE (10% gel) and Coomassie staining.

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was added to a final concentration of 0.4 mM, and the cells were incubated for an additional 1 h at 25 °C. Cells were lysed by sonication in Buffer A containing 50 mM potassium phosphate, pH 7.5, 50 mM KCl, 0.1% Triton X-100, and Complete protease inhibitor mixture (Roche Molecular Biochemicals). The lysate was cleared by centrifugation and incubated at 4 °C for 1 h with phosphocellulose resin (Whatman) previously equilibrated with Buffer A. Following incubation, the resin was washed extensively with Buffer A, and the proteins were eluted with Buffer A plus 300 mM KCl. The eluate was mixed with cobalt-chelating TALON-Sepharose (Clontech) equilibrated with Buffer A. The resin was washed with 10 column volumes of Buffer A plus 300 mM NaCl, and the protein was eluted with Buffer A containing 150 mM imidazole and 150 mM KCl. The eluate was mixed with anti-FLAG monoclonal antibody-conjugating Sepharose (Sigma) equilibrated with Buffer A for 2 h at 4 °C. Following incubation, the resin was washed extensively with Buffer A, and the proteins were eluted with Buffer A plus 0.1 mg/ml FLAG peptide. Finally, the proteins were applied to a Superdex 200 200 PC2 3/30 SMART column (Amersham Biosciences) equilibrated with Buffer A containing 150 mM KCl.

Deletion mutants with the GST tag were purified according to the manufacturer's instructions (Amersham Biosciences). T7- and His6-tagged mouse geminin was expressed in bacteria and purified to near homogeneity by cobalt-chelating TALON-Sepharose (Clontech) on a MiniS SMART column followed by a Superdex 200 PC2 3/30 SMART column according to instructions provided by the manufacturer (Amersham Biosciences). Purification of the recombinant mouse MCM4/6/7 complex in insect cells was performed as described previously (13).

**Antibody**—Polyclonal antibody was prepared from a rabbit injected
with the carboxyl-terminal fragment of bacterially expressed mCdt1 (amino acids 425–557). A PCR fragment containing this region was digested by EcoRI and BamHI and subcloned into EcoRI- and BamHI-digested pET24a (Novagen). PCR primers were as follows: 5′-CCGAAT
TCAGGGCCTGGAAGGGTTGTC3′ and 5′-CCGCTACCTCGAGCCCTCGGCTTGGCACT-3′. Antibody was purified by Thiophilic-~Unif~ resin (Clontech) according to the manufacturer’s instructions.

Competition Precipitation Assay—Fifty ng of mCdt1 and 50 ng of MCM4/6/7 complex were mixed with glutathione-agarose (Amersham Biosciences) for 4 h at 0 °C in 100 μl of NET-gel buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, and 0.25% gelatin. After washing with NET-gel buffer, precipitates were dissolved with 30 μl of 2X Laemmli sample buffer and then subjected to SDS-PAGE and Western blot analysis using anti-GST (Amersham Biosciences), anti-mCdt1, and anti-geminin (Santa Cruz Biotechnology), and anti-mCdt1 antibodies as described previously (31).

**Gel Mobility Shift Assay**—Fifty ng of GST-tagged mCdt1 was incubated with 20 μl of single-stranded DNA-cellulose or double-stranded DNA-cellulose (Sigma), which had been equilibrated with Buffer A for 1 h at 4 °C. After washing with Buffer A, bound proteins were eluted by increasing concentrations of NaCl. Proteins were subjected to SDS-PAGE followed by silver staining. To identify the mCdt1 domain essential for DNA binding, full-length mCdt1 was mixed simultaneously with five amino-terminally truncated proteins (GSTAN1–127, GSTAN1–176, GSTAN1–293, GSTAN1–406, and GSTAN1–478) or three carboxyl-terminally truncated proteins (GSTAC381–557, GSTAC180–557, and GSTAC131–557) and incubated with DNA-cellulose. Bound proteins were eluted by increasing concentrations of NaCl. Proteins were detected by silver staining. To examine the effect of geminin on the DNA binding activity of mCdt1, increased amounts of T7-tagged geminin were preincubated with 50 ng of GST-tagged mCdt1 for 1 h at 4 °C and incubated with double-stranded DNA-cellulose for 1 h at 4 °C, and unbound proteins were subjected to SDS-PAGE followed by Western blot analysis with an anti-mCdt1 and anti-geminin antibodies.

**Gel Mobility Shift Assay**—Sequences of the oligonucleotides used are as follows: 17-mer, 5′-TGGTTCCCATCAGCAGC-3′; 37-mer, 5′-GGTTCTCCGATCTCAGCAGTGGTAAAGCAAGCCGGCAGT-3′; 70-mer, 5′-GGTTTCTCCGATCTCAGCAGTGGTAAAGCAAGCCGGCAGTCTGGACACGCTGCGTACCTAG-3′; 70-mer (AT), 5′-TTTTTTATGGTAGATCTTTTATGCTTGCTTTTCAAAAGGCCTGCAGGCAAGTGCCACAGTGCCAC-3′; and 5′-GAAGGTGACGGATCCGATGCTACCTAGA3′. The gel-purified oligonucleotides were 5′-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. 32P-End-labeled oligonucleotide (50 fmol) and 50 ng of FLAG-tagged mCdt1 were incubated for 30 min at 37 °C in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mg/ml bovine serum albumin, 5% glycerol, 0.05% Triton X-100, and 1 mM dithiothreitol) in a 10-μl reaction volume. Protein-DNA complexes were resolved on 1% agarose gels at 4 °C in 0.5× TBE buffer (45 mM Tris-HCl, 45 mM H3BO3, 10 mM EDTA, pH 8.3) at 10 V. After electrophoresis, the gel was fixed, dried, and subjected to autoradiography. For super-shift assays, anti-T7 or anti-FLAG M2 (Sigma) antibody was added to the above reaction, and the mixture was incubated on ice for 10 min before loading on gels.

**RESULTS**

cDNA Cloning of Mouse Cdt1—To identify the cDNA encoding the mouse Cdt1, we screened the mouse EST database and obtained the clones AI605978, AA139554, and AA671429. The full-length open reading frame was obtained by screening a mouse embryonic fibroblast cDNA library (Clontech). The cloned cDNA encodes a putative protein of 557 amino acid residues with a calculated molecular mass of 61.5 kDa. The predicted protein is 72% identical and 79% similar to human Cdt1, 46% identical to Xenopus Cdt1, and 25% identical to Drosophila Cdt1/DUP. Alignment of the mCdt1 cDNA sequence with the human, Xenopus, Drosophila, S. pombe, and S. cerevisiae sequences reveals extensive conservation in the carboxy-terminal region of the proteins for a broad range of eukaryotes (Fig. 1). The central region, spanning 200 to 350 residues, is conserved among metazoans but is not found in fungi. Canonical sequence motifs were not detected for the mouse sequence. We also screened genomic sequence databases with the mCdt1 cDNA and found genomic sequences containing mCdt1 open reading frames. The full-length mCdt1 open reading frame consists of 10 exons interrupted by 9 introns and overall spans 4588 bases (data not shown). All exon-intron junction sequences conform to the canonical GT/AG rule. The positions of introns are depicted by arrowheads in Fig. 1B. Interestingly, the central region conserved among metazoans is
encoded exactly by exons 4 and 5, whereas the carboxyl-terminal region conserved among eukaryotes is encoded by exons 9 and 10. While our manuscript was in preparation, cDNA encoding mouse Cdt1 homologue was reported (32). The coding sequence of our mCdt1 cDNA was identical to that of the mCdt1 gene published recently (32).

**Yeast Two-hybrid Analysis**—To analyze mCdt1 protein-protein interactions, we carried out a yeast two-hybrid analysis. Full-length mCdt1 was fused to the LexA DNA binding domain as a bait, and various replication factors, including mouse ORC1–6, MCM2–7, Cdc6, Cdc45, and geminin, were fused to the BA42 transcriptional activation domain as prey, and interactions between bait and prey were monitored in yeast cells. We found that mCdt1 associates tightly with mouse MCM6 and mouse geminin (Fig. 2A). In addition, a weak but significant interaction of mCdt1 with mouse ORC2 was also detected. To further determine which domains are required for interactions with MCM6 and geminin, an mCdt1 deletion series was constructed as baits, as shown in Fig. 2B, and two-hybrid analyses were performed. The amino-terminally truncated mutant ΔN1–171 and the carboxyl-terminally truncated mutant ΔC520–557 strongly interact with both geminin and Mcm6, as efficiently as does full-length mCdt1. The interaction involving the ΔC520–557 construct reproducibly yielded higher β-galactosidase activity than did interactions involving full-length mCdt1, suggesting that the carboxyl-terminal region (521–557) may inhibit the binding of mCdt1 to geminin or MCM6. In contrast, mutant constructs with larger deletions (ΔN1–458 and ΔC131–557) are not capable of binding to MCM6 and geminin fusions. Interestingly, the amino-terminally truncated mutant ΔN1–334 associates with MCM6 but not with geminin, whereas the carboxyl-terminally truncated mutant ΔC289–557 associates with geminin but not with MCM6. These results suggest that the central region (residues 172–288) is important for interaction with geminin, whereas the carboxyl terminus (residues 335–519) is required for interaction with MCM6. These results were confirmed by in vitro binding experiments as described below (see Fig. 4).

**Expression and Purification of Bacterially Expressed mCdt1**—The cDNA encoding mCdt1 was inserted into the prokaryotic expression vector pET24a and flanked with sequences encoding the FLAG peptide at the amino terminus, and the His tag at the carboxyl terminus (Fig. 3A). This construct was introduced into BL21(DE3). Expression of recombinant proteins was induced by isopropyl-β-D-thiogalactopyranoside, and expressed proteins were purified by sequential
hybrid analyses were also fused with the GST tag (Fig. 3).

Several deletion mutants that purified as shown in Fig. 3 were designed based on information obtained from yeast two-hybrid analyses were also fused with the GST tag (Fig. 3A) and purified as shown in Fig. 3C. Using these purified proteins, we examined the interactions of mCdt1 with geminin, MCM6, and DNA.

Co-immunoprecipitation of mCdt1 with geminin and the MCM4/6/7 Complex—To confirm physical interactions between mCdt1 and geminin or MCM6, as suggested by the yeast two-hybrid analysis, we performed a co-immunoprecipitation analysis using an anti-mCdt1 antibody. Anti-mCdt1 antisera was raised against a bacterially expressed and purified carboxyl-terminal fragment of mCdt1 that spans residues 425 to 557. We found that among eight deletion mutants, geminin was associated with either single-stranded or double-stranded DNA-cellulose as shown in Fig. 3B. FLAG- and His<sub>6</sub>-tagged mCdt1 was purified as a 65-kDa protein to near homogeneity. Several deletion mutants that were designed based on information obtained from yeast two-hybrid analyses were also fused with the GST tag (Fig. 3A) and purified as shown in Fig. 3C. Using these purified proteins, we examined the interactions of mCdt1 with geminin, MCM6, and DNA.

DNA-cellulose Binding Assay—During the purification of mCdt1 proteins, we noticed that mCdt1 associates with single-stranded DNA-cellulose. Fractionation of mCdt1 was performed using either single-stranded DNA-cellulose or double-stranded DNA-cellulose. In the presence of 50 mM NaCl, mCdt1 associates with both single- and double-stranded DNA-cellulose. We found that among eight deletion mutants, geminin was associated with the MCM4/6/7 complex and geminin was precipitated with amino-terminally and carboxyl-terminally truncated mCdt1 mutants (GST-ΔN1–127, GST-ΔN1–176, and GST-ΔC381–557), whereas the MCM4/6/7 complex was found to be associated with the amino-terminally truncated mCdt1 mutants (GST-ΔN1–127, GST-ΔN1–176, GST-ΔN1–176, and GST-ΔN1–176–293, and GST-ΔN1–406). These results are consistent with the previous results obtained by yeast two-hybrid analysis indicating that the central region (residues 177–380) and the carboxyl-terminal region (residues 407–477) are binding sites for geminin and MCM6, respectively. The amino-terminal region (1–176) is dispensable for interactions with geminin or MCM6.

Mouse Cdt1 Associates with Geminin, MCM6, and DNA
interaction of mCdt1 with double-stranded DNA in a dose-dependent manner (Fig. 5B).

To determine which domains are required for interaction with single-stranded or double-stranded DNA-cellulose, a series of mCdt1 deletion proteins was subjected to DNA-cellulose binding analysis. To compare the relative affinities of the deletion proteins for DNA-cellulose with that of full-length mCdt1, GST-tagged full-length mCdt1 and four amino-terminally truncated mutant proteins (GST–ΔN1–127, GST–ΔN1–176, GST–ΔN1–293, and GST–ΔN1–406) or three carboxyl-terminally truncated mutant proteins (GST–ΔC131–557, GST–ΔC180–557, and GST–ΔC381–557) were simultaneously mixed with either single-stranded DNA-cellulose (left panel) or double-stranded DNA-cellulose (right panel) at 50 mM NaCl for 1 h at 4 °C and eluted by increasing concentrations of NaCl. Proteins were detected by silver staining. GST fusion proteins are depicted by asterisks. D, schematic representation of the domain organization of mCdt1. Interaction domains required for binding to geminin, MCM6, and DNA are summarized.

**Electrophoretic Mobility Shift Assay of mCdt1**—To confirm the ability of mCdt1 to bind to DNA, we performed electrophoretic mobility shift assays. 32P-End-labeled oligonucleotides with lengths of 17, 37, or 70 bases were incubated with purified mCdt1, and bound proteins were detected by agarose gel electrophoresis followed by autoradiography. Incubation of mCdt1 with the 70- and 37-mer results in the formation of a distinct complex of altered mobility, suggesting that mCdt1 associates tightly with the 37- and the 70-mer in a dose-dependent manner (Fig. 6A). In sharp contrast, mCdt1 causes only a slight increase in the mobility of the 17-mer, indicating that mCdt1 cannot efficiently associate with the 17-mer and that it prefers longer single-stranded oligonucleotides. mCdt1 incubated with DNA does not enter a 4% acrylamide gel, suggesting that the mCdt1-DNA complex is a large network (data not shown).
FIG. 6. **Electrophoretic mobility shift assay of mCdt1.** A, various amounts of mCdt1 were incubated with 5 fmol of $^{32}$P-labeled oligonucleotides (17, 37, or 70 bases in length) for 30 min at 37°C. Protein-DNA complexes were analyzed by 1% native agarose gel electrophoresis in 0.5× TBE. Gels were dried, exposed on imaging plates, and analyzed with a BAS2500 Fuji image analyzer. Right and left panels show single-stranded oligonucleotide (ssDNA) and double-stranded oligonucleotide (dsDNA), respectively. B, competition assay of mCdt1 with various DNA substrates. Fifty ng of mCdt1 was incubated with 5 fmol of a $^{32}$P-end-labeled 70-mer in the presence of various amounts of DNA substrates as indicated, and protein-DNA complexes were analyzed by agarose gel electrophoresis. AT indicates a 70-base oligonucleotide containing an ARS1 sequence. M13mp18, pUC18(CC), and pUC18(L) indicate single-stranded M13 plasmid DNA, covalently closed circular double-stranded pUC DNA, and Smal-digested double-stranded linear pUC18 plasmid DNA, respectively. C, full-length mCdt1 (FLAG-mCdt1-His$_6$ (FLAG-FL, lane 2)) and various
noteworthy that the mobility-shifted bands are heterogeneous in size. With lower amounts of mCdt1, a slight shift in mobility is observed with the 37- and the 70-mer. When increased amounts of mCdt1 are added, complexes that migrate even more slowly than the initial specific complex are observed. Thus, a multimeric mCdt1 complex might form in the presence of DNA in a dose-dependent fashion.

Next, we performed competitive electrophoretic mobility shift assays. The $^{32}$P-end-labeled 70-base oligonucleotide was mixed with various amounts of the unlabeled 70-mer, an AT-rich 70-mer containing the S. cerevisiae ARS1 sequence, single-stranded M13 plasmid DNA, covalently closed circular double-stranded pUC DNA, or Smal-digested double-stranded linear pUC18 plasmid DNA (Fig. 6B). In the presence of the unlabeled 70-mer, the association of mCdt1 with the $^{32}$P-end-labeled 70-mer is unchanged, consistent with the previous result (Fig. 6A) that association with a $^{32}$P-labeled 17-mer is less efficient. In contrast, other substrates, including the 70-mer, single-stranded DNA, covalently closed circular DNA and linearized double-stranded DNA compete efficiently with the $^{32}$P-labeled 70-mer. Hence, these results indicate that mCdt1 associates with DNA in a sequence-nonspecific, strand-nonspecific, and conformation-nonspecific manner. We also found that poly(T), salmon sperm DNA, and activated calf thymus DNA compete efficiently with the $^{32}$P-labeled oligonucleotide, whereas yeast transfer RNA or DNA that is treated with high concentrations of ethidium bromide do not (data not shown).

To confirm our conclusion that the amino-terminal region of mCdt1 is a binding domain with DNA, an electrophoretic mobility shift assay with deletion mutant proteins was carried out. The amino-terminally truncated protein (GSTΔN1–293) and the carboxy-terminally truncated proteins (GSTΔC381–557 and GSTΔC131–557) were mixed with $^{32}$P-end-labeled 70-base oligonucleotide, and bound proteins were detected by agarose gel electrophoresis. As shown in Fig. 6C, incubation of the probe with GSTΔC381–557 and GSTΔC131–557 results in the formation of a distinct complex of altered mobility, whereas, GSTΔN1–293 causes only a slight increase in the mobility of 70-mer. These results clearly indicate that the amino-terminal region of mCdt1 (residues 1–293) is responsible for binding to DNA.

Finally, the effect of geminin on the DNA binding activity of mCdt1 was examined. The $^{32}$P-end-labeled 70-mer was mixed with mCdt1 in the presence of various amounts of geminin, and a complex with reduced mobility was detected as shown in Fig. 6D. In the presence of geminin, the intensity of this signal is severely reduced, indicating that geminin inhibits the interaction of mCdt1 with DNA. These results are consistent with our finding (Fig. 5B) that geminin inhibits the interaction of mCdt1 with DNA-cellulose. When excess amounts of geminin were mixed with mCdt1, we observed a distinct shifted complex as shown in Fig. 6D, lanes 4 and 5 (asterisk). This minor signal represents a geminin-mCdt1 complex, because the signal is super-shifted by incubation of the complex with either anti-geminin or anti-mCdt1 antibodies (data not shown), suggesting that the geminin-mCdt1 complex also has a weak affinity for single-stranded DNA. However, most of the probe is observed at the bottom of the gel, indicating that the principal effect of geminin is to inhibit the binding of mCdt1 to DNA.

### DISCUSSION

In this study, we identified domains and novel biochemical properties of the mouse Cdt1 protein. mCdt1 associates with geminin and the MCM4/6/7 complex via the central region, which spans amino acid residues 177–380, and the carboxy-terminal region, which spans residues 407–477. In addition, mCdt1 is capable of binding to DNA through its amino-terminal region (residues 1–293). Although mCdt1 prefers longer oligonucleotides such as a 70-mer to the shorter 17-mer, it efficiently binds to DNA in a sequence-nonspecific, strand-nonspecific, and conformation-nonspecific manner. Geminin inhibits the ability of mCdt1 to bind both MCM and DNA. These findings will provide new insights for understanding the molecular basis of the initiation of replication.

**Mechanism of the Inhibition of Cdt1 Activities by geminin—**

Our yeast two-hybrid analysis and deletion studies with bacterially expressed and purified proteins revealed that the central region and the carboxy-terminal region of mCdt1 play roles in binding to geminin and MCM6, respectively. To date, geminin, a specific inhibitor of Cdt1, has been found only in metazoans but not in S. cerevisiae and S. pombe. Interestingly, the carboxy-terminal region of Cdt1 is conserved among all eukaryotes, including S. cerevisiae and S. pombe, whereas the central region is only conserved among metazoans, including Drosophila, mice, and humans (Fig. 1B). These results suggest that the Cdt1 domain that associates with the MCM4/6/7 complex is conserved among all eukaryotes, whereas the geminin binding domain is conserved only among metazoans.

Although geminin is used widely to inhibit the loading of the MCM complex onto Xenopus chromatin, the mechanism by which geminin inhibits the assembly of the pre-RC is not fully understood (1, 8, 16). In the presence of geminin, interactions between not only mCdt1 and MCM6 but also between mCdt1 and DNA are disturbed severely (see Figs. 4B and 5B). How does geminin inhibit the binding of Cdt1 to MCM and DNA? The mCdt1 DNA binding region was found to overlap with the geminin binding domain, which is in the central region (177–380). Thus, geminin could mask the DNA binding region by tightly interacting with the mCdt1 central region. However, we have found that the geminin and MCM interaction domains are different. It is tempting to speculate that the association of geminin with the mCdt1 central region causes a conformational change in the overall structure of mCdt1, which concomitantly causes the carboxy-terminal binding domain to be masked. To clarify the precise mechanism by which geminin regulates Cdt1, structural information will prove useful in elucidating how Cdt1 and geminin interact at the protein level.

**The Architecture of the Pre-RC—**

Previously, we found specific interactions between human MCM10 and human ORC2 or mouse MCM6 by the yeast two-hybrid technique and by co-immunoprecipitation analysis (30). Recently, human MCM10 was found to be expressed at the G1/S boundary and to accumulate during S phase (35). In contrast, human Cdt1 accumulates in G phase and rapidly diminishes in S phase (25, 33, 34). Because the timing of expression of human Cdt1 and human MCM10 are mutually exclusive and because both Cdt1 and MCM10 associate successively with ORC2 and MCM6, it is interesting to speculate that Cdt1 is replaced by MCM10 at the G1/S phase boundary and that this change reflects the transi-
tion of the pre-RC to an initiation complex, as predicted by Bell and Dutta (1).

Cdt6 has been considered to function as a clamp loader for MCM, because it exhibits similarity to the bacterial clamp loader DnaC (17). After DnaC loads replicative DnaB helicase onto the origin, DnaC plays a additional role in anchoring the replicative DnaB helicase at the origin until the onset of replication, which is mediated by a cryptic single-stranded DNA binding activity (34). In this report, we found that mCdt1 possesses single-stranded DNA binding activity. In addition, it has been reported that Cdt1 assembles onto the pre-RC prior to loading MCM during G1 phase, and some initiator proteins such as DnaA, and DNA binding activity of Cdt1 may contribute to anchoring the MCM helicase at the origin during S phase (25, 29, 34). Therefore, it is tempting to speculate that the DNA binding activity of Cdt1 may contribute to modulate chromatin structure (37, 38). Although the initiation, which is mediated by a cryptic single-stranded DNA binding activity using recombinant MCM2–7, Cdc6, Cdt1, and a physiological template that includes nucleosomes or the nuclear matrix, a fact that has not been achieved to date. The protein–protein interactions that we have identified between Cdt1 and components of the pre-RC will be useful in the in vitro reconstitution of the pre-RC formation.

mCdt1 Is a DNA Binding Factor—mCdt1 associates with oligonucleotides longer than 37 bases, double-stranded covalently closed circular DNA, single-stranded circular DNA, poly(dT), and linearized double-stranded DNA. Sonicated salmon sperm DNA and activated calf thymus DNA also compete efficiently with the binding of mCdt1 to a 32P-labeled salmon sperm DNA and activated calf thymus DNA also compete efficiently with the binding of mCdt1 to a 32P-labeled single-stranded DNA binding activity, it may be a good candidate for a putative initiation factor. To test the possibility that multimerized mCdt1 may have a DNA unwinding activity, we assayed its effect on relaxed covalently closed circular DNA as a substrate (43). However, we were unable to detect such an activity using purified mCdt1 protein and covalently closed circular plasmid (data not shown). Future studies will be necessary to define the role of the mCdt1 DNA binding activity at the onset of replication. Localization of mCdt1 in the nucleus, the effect of mCdt1 on the DNA binding activity of ORC, and its effect on MCM helicase activities with respect to physiological substrates should provide new insights into the molecular basis of eukaryotic replication.

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