

## Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells

ZHEN YAN\*, JAMES DE GREGORI†, RALPH SHOHEET\*, GUSTAVO LEONE†, BRUCE STILLMAN‡, JOSEPH R. NEVINS†, AND R. SANDERS WILLIAMS\*§

\*University of Texas Southwestern Medical Center, Dallas, TX 75235; †Duke University Medical Center, Durham, NC 27710; and ‡Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

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**ABSTRACT** Cdc6 has a critical regulatory role in the initiation of DNA replication in yeasts, but its function in mammalian cells has not been characterized. We show here that Cdc6 is expressed selectively in proliferating but not quiescent mammalian cells, both in culture and within tissues of intact animals. During the transition from a growth-arrested to a proliferative state, transcription of mammalian Cdc6 is regulated by E2F proteins, as revealed by a functional analysis of the human Cdc6 promoter and by the ability of exogenously expressed E2F proteins to stimulate the endogenous Cdc6 gene. Immunodepletion of Cdc6 by microinjection of anti-Cdc6 antibody blocks initiation of DNA replication in a human tumor cell line. We conclude that expression of human Cdc6 is regulated in response to mitogenic signals through transcriptional control mechanisms involving E2F proteins, and that Cdc6 is required for initiation of DNA replication in mammalian cells.

In yeasts, Cdc6 (*Saccharomyces cerevisiae*) and Cdc18 (*Schizosaccharomyces pombe*) associate with the origin recognition complex (ORC) proteins to render cells competent for DNA replication (1–10). The association of Cdc6 with ORC alters the nucleotide contacts made by origin binding proteins (11) and promotes recruitment of MCM proteins and other factors required to form prereplication complexes (12, 13). Initiation of DNA replication also requires the activity of S-phase promoting protein kinases that include cyclin:cdk and Cdc7:Dbf4 complexes (1).

Cdc6-related proteins recently have been characterized from both human (14) and *Xenopus* cells (14, 15), but only limited information is available with respect to the expression and function of Cdc6 in higher eukaryotes. *Xenopus* Cdc6 was shown to bind ORC and to promote assembly of MCM proteins onto chromatin. In addition, immunodepletion of Cdc6 blocked initiation in a cell free replication assay that uses *Xenopus* oocyte extract and sperm chromatin (15). We have reported that human Cdc6 mRNA and protein were not detected in serum-deprived human diploid fibroblasts, and that expression of human Cdc6 was induced, prior to the G<sub>1</sub>/S transition, when these cells were stimulated by serum to enter the cell cycle (14).

In this study, we addressed several questions. Is the association between Cdc6 expression and proliferative cell growth evident in a different cell culture model of growth arrest (contact inhibition versus serum starvation), and in tissues of intact animals? What regulatory mechanisms act upstream to control expression of Cdc6? Is Cdc6 required for initiation of DNA replication in mammalian cells? The results presented here demonstrate that mammalian Cdc6 is subject to strict

regulation in response to mitogenic growth signals through mechanisms that involve E2F transcription factors. The timing of Cdc6 expression in the transition from G<sub>0</sub> to G<sub>1</sub> is consistent with its postulated role in DNA replication, and immunodepletion of Cdc6 blocks entry of human cells into S phase. These results support the hypothesis that human Cdc6, like its yeast counterparts, participates in assembly of prereplication complexes. In addition, these data indicate that Cdc6, a protein likely to function directly at origins of DNA replication, is a downstream target of molecular signaling pathways that control the G<sub>1</sub>/S transition.

### MATERIALS AND METHODS

**Analysis of Cdc6 Expression.** Immunoblots were performed as described (16) using purified rabbit polyclonal antibodies generated in our laboratory against human Cdc6, or other antibodies obtained from commercial sources [cyclin A (Santa Cruz Biotechnology), PCNA (Calbiochem), cdc2 (Transduction Laboratory, Lexington, KY), and Sp1 (Santa Cruz Biotechnology)]. Northern hybridizations were performed by standard procedures using a cloned human Cdc6 cDNA as the probe (14). Indirect immunofluorescence of cultured human cells was performed as described (17) using rabbit anti-human Cdc6 as the primary antibody and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate as the secondary antibody. *In situ* hybridizations were performed as described (18, 19) on paraffin sections of duodenum from adult mice. Species-specific, radiolabeled probes were generated by *in vitro* transcription in the presence of <sup>33</sup>P-UTP from a DNA template containing a murine Cdc6 gene fragment corresponding to nucleotides 983–1184 of human Cdc6 cDNA (14).

**Analysis of Human Cdc6 Promoter Activity.** We previously cloned the human Cdc6 gene from a chromosome 17-specific cosmid library (14) and determined the transcriptional start site by primer extension analysis of HeLa cell mRNA. This position corresponded to the 5' terminus of the longest Cdc6 cDNA clones that could be isolated from a HeLa cell library or by 5' rapid amplification of cDNA ends using mRNA from human 293 cells as the template. A *KpnI*-*SacI* DNA fragment encompassing the human Cdc6 promoter region (–1,700 to +7) was ligated to the firefly luciferase gene in pGL3 (Promega). Electrophoretic mobility shift assays were performed as described (16) to assess binding of E2F proteins to a double-stranded deoxyoligonucleotide representing putative E2F binding motifs from wild-type or mutated Cdc6 promoter sequences.

Using a PCR-mediated mutagenesis method (20), nucleotide substitutions were introduced into both of the two putative E2F binding sites at nucleotide positions –43 to –36 and –8 to –1 relative to the transcriptional start site. Specifically, the

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Abbreviation: ORC, origin recognition complex.

§University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, NB11.200, Dallas, TX 75235-8573. e-mail: williams@ryburn.swmed.edu.

sequence motifs TTTGGCGG and TTTGGCGC were converted to AATTCGG and AAAGCTTG, respectively. Plasmids bearing wild-type or mutated human Cdc6 promoter sequences were transfected into NIH 3T3 cells by using Lipofectamine (GIBCO/BRL). The efficiency of transfection was internally controlled by cotransfection of a pCMV-lacZ expression plasmid. After transfection, subconfluent cells were arrested from proliferative growth by serum starvation (0.1% fetal bovine serum in DMEM) for 36 hr, and harvested at various intervals following readdition of serum (10% fetal bovine serum in DMEM). Whole cell lysates were prepared and assayed for luciferase and  $\beta$ -galactosidase activity.

**Recombinant Adenoviral Vectors.** Complementary DNA sequences encoding E2F1, E2F2, E2F3, or DP1 under the control of a cytomegalovirus promoter were packaged into infectious virions following homologous recombination into an E1A-deleted Ad5 genome in 293 cells. A virus (Ad-Con) lacking an insert in the cytomegalovirus expression cassette was employed as a negative control. Recombinant virions were purified by cesium chloride gradient density centrifugation and titered by an immunofluorescence assay detecting expression of the viral 72-kDa DNA binding protein (21).

**Immunodepletion of Cdc6 in Human Cells.** Rabbit polyclonal antibodies were raised against a fragment of human Cdc6 (amino acids 51–172), and purified by ammonium sulfate precipitation, followed by affinity chromatography over a column prepared by linking the immunogen to Sepharose beads (Affi10 column, Bio-Rad). The specificity of the anti-Cdc6 antibody preparation was established by immunoblot, immunofluorescence and immunoprecipitation assays. Purified anti-Cdc6 antibody produced a nuclear immunofluorescence pattern in cycling human HeLa cells that was unaffected by preabsorption against immobilized glutathione *S*-transferase. In contrast, preabsorption against an immobilized human Cdc6-glutathione *S*-transferase fusion protein eliminated the ability of purified antiserum to detect a nuclear fluorescence signal in cycling human cells. In immunoblot assays of nuclear protein extracts of HeLa cells transfected to express either full length Cdc6 or a truncated Cdc6 variant (amino acids 51–172) tagged with an influenza hemagglutinin antigen epitope, both anti-hemagglutinin antigen and anti-Cdc6 antibodies detected the same protein bands of the predicted molecular sizes. Affinity-purified anti-Cdc6 antibody was concentrated to 1.5  $\mu\text{g}/\mu\text{l}$  in 0.5 $\times$  PBS, and micro-injected into metaphase cells identified by phase contrast microscopy from among a population of subconfluent HeLa cells growing asynchronously on a photo-etched coverslip (Bellco Glass) in growth medium (DMEM supplemented with 10% fetal bovine serum). Cells were incubated subsequently in growth medium containing 10  $\mu\text{M}$  5-bromodeoxyuridine (BrdUrd) for 15–18 hr, and the percentage of cells undergoing DNA replication was determined after immunostaining by using a mouse anti-BrdUrd mAb (Boehringer Mannheim). Injected cells were identified by immunofluorescence using goat anti-rabbit IgG conjugated to fluorescein isothiocyanate.

## RESULTS

**Expression of Cdc6 in Mammalian Cells and Tissues.** We demonstrated previously that expression of human Cdc6 mRNA and protein was suppressed by serum starvation of cultured human diploid fibroblasts, and stimulated by the readdition of serum (14). To determine whether intracellular concentrations of Cdc6 are regulated in a similar manner by other stimuli that control cell proliferation, we examined the expression of Cdc6 in human T24 cells subjected to growth arrest by contact inhibition in the presence of serum by immunoblot and immunofluorescence techniques. During the transition from  $G_0$  into the cell cycle, Cdc6 protein was first detected about 4 hr prior to the initiation of S phase (Fig. 1).

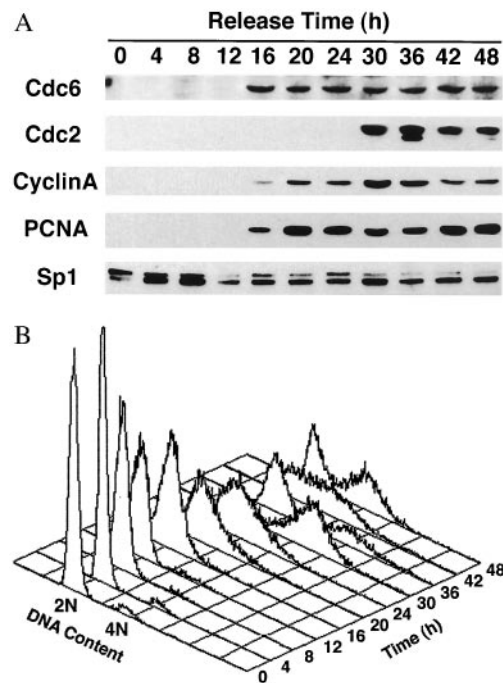


FIG. 1. Induction of Cdc6 in human cells during release from contact inhibition. (A) Immunoblot analysis of Cdc6 expression in relation to other proteins at specific time points during entry into the cell cycle. Human T24 cells were plated at high density ( $4 \times 10^6$  cells/150 mm) in McCoy 5A media supplemented with 10% fetal bovine serum and grown for 4 days. To release cells from contact inhibition, they were trypsinized and replated at lower density ( $1 \times 10^6$  cells/150 mm) in the same media. (B) Flow cytometric analysis of DNA content in T24 cells samples at specific time points after release from contact inhibition. The results show that contact-inhibited cells are arrested with unreplicated DNA (2N), and that cells enter S phase between 20 and 24 hr after release.

The time course of Cdc6 induction was contemporaneous with that of PCNA, but preceded that of cyclin A and cdc2. In the immunofluorescence analysis, Cdc6 was localized predominantly to the nuclei of asynchronously cycling T24 cells, but could not be detected in contact-inhibited cells (Fig. 2).

The pattern of Cdc6 expression in cells of intact mammalian tissues was assessed by *in situ* hybridization studies of mouse tissues. Within normal intestinal epithelium, DNA replication is limited to the crypts of Lieberkühn, while differentiated epithelial cells that line the villi extending into the intestinal lumen are quiescent. Expression of Cdc6 mRNA is evident only within the proliferating cell population of this tissue (Fig. 3), a finding consistent with its postulated function in the initiation of DNA synthesis.

**Regulation of Cdc6 Transcription by E2F Proteins in Mammalian Cells.** The prominent role of E2F transcription factors in preparing cells for DNA replication (22), and the presence of putative E2F binding sites within the proximal promoter region of the human Cdc6 gene (14), suggested that Cdc6 gene transcription could be regulated by E2F proteins.

We employed recombinant adenoviruses to determine whether forced expression of E2F proteins could stimulate transcription of the Cdc6 gene in growth arrested human cells. Under conditions of serum deprivation, human foreskin fibroblasts expressed only low levels of Cdc6 mRNA, but forced expression of either E2F1, E2F2, or E2F3, together with their heterodimeric partner DP1, stimulated the accumulation of Cdc6 mRNA in these quiescent human cells (Fig. 4A). This induction of human Cdc6 expression was specific to adenoviruses expressing E2F:DP1 proteins, and was not observed in cells infected with comparable titers of a control virus.



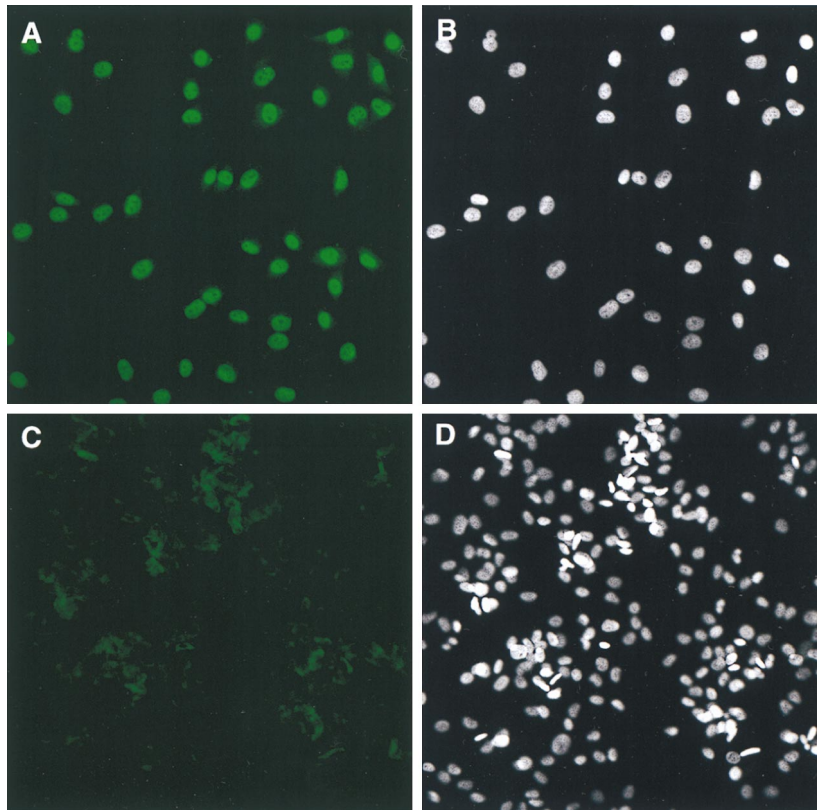


FIG. 2. Immunofluorescence staining of Cdc6 in proliferating human cells. Cdc6 is localized to the nucleus of asynchronously proliferating T24 cells (A) but is not detected in contact-inhibited cells (C). Propidium iodide staining (Right) identifies all the nuclei present within the same fields (B and D).

To determine whether direct binding of E2F proteins to the human Cdc6 promoter is pertinent to the control of Cdc6 transcription, a genomic fragment including the human Cdc6 promoter (nucleotides  $-1,700$  to  $+7$  relative to the transcriptional start site) was linked to a luciferase reporter gene and

introduced by transfection into murine 3T3 cells. Under conditions of growth arrest induced by serum deprivation, this promoter was inactive, corresponding to low or undetectable levels of endogenous Cdc6 mRNA (Fig. 4B). Addition of serum, allowing 3T3 cells to reenter the cell cycle, increased

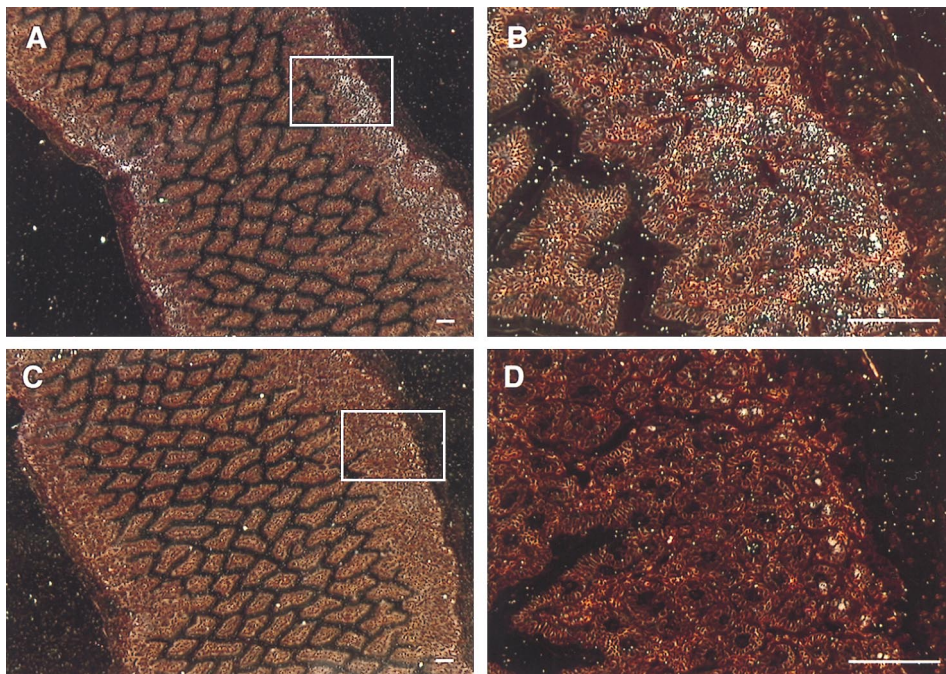


FIG. 3. Selective expression of Cdc6 in proliferating cell populations within intact mammalian tissues. *In situ* hybridization of sections of duodenum from an adult mouse by using antisense (A and B) or sense (C and D) probes, transcribed *in vitro* from Cdc6 cDNA. The boxed areas in A and C are shown at higher magnification in B and D. (Bar = 100  $\mu\text{m}$ .)

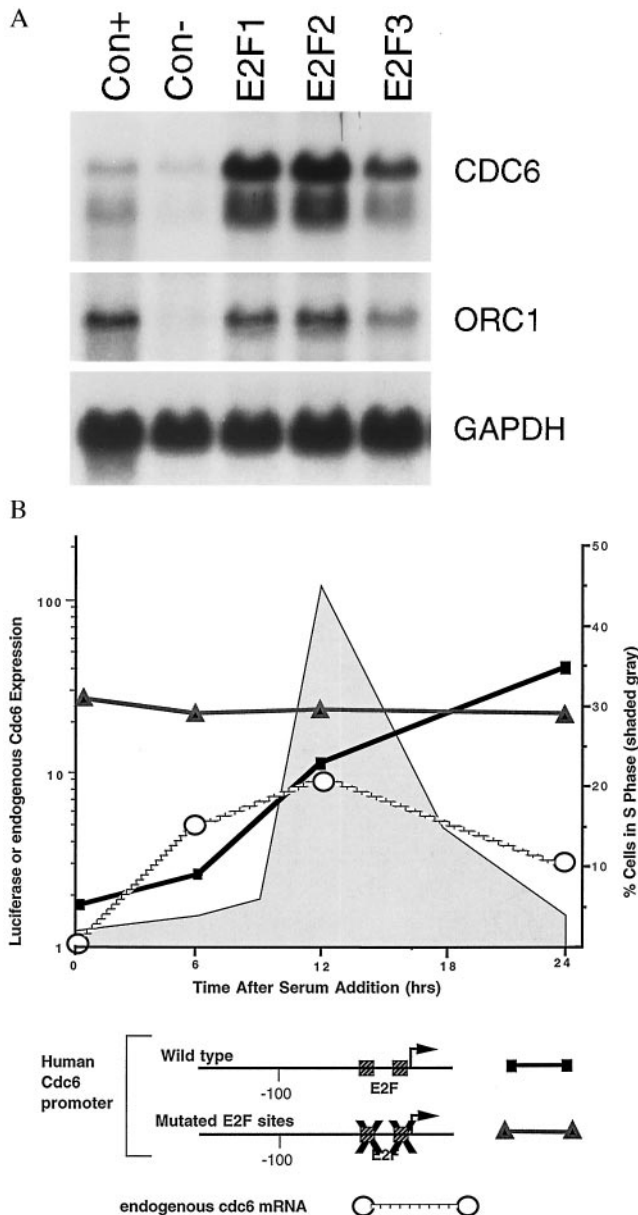


FIG. 4. (A) Quiescent, serum-deprived human foreskin fibroblasts (passage 7) were infected with the indicated recombinant adenoviruses, each at a multiplicity of infection of 300, and then harvested for Northern blot analysis at 15 hr postinfection. Blots were hybridized with a human Cdc6 probe, or with a glyceraldehyde-3-phosphate dehydrogenase probe as a control for RNA loading. The samples labeled as E2F1, E2F2, or E2F3 represent infections with the indicated E2F viruses together with an adenovirus expressing DP1 to provide the dimeric partner for E2F. (B) E2F binding sites are required for correct regulation of the human Cdc6 promoter. Transient transfection assays were performed in subconfluent 3T3 cells under conditions of growth arrest induced by serum deprivation ( $t = 0$  hr), and at subsequent time points after readdition of serum. Reporter gene activity (mean of triplicate determinations) was calculated relative to expression of a cytomegalovirus-luciferase gene in sister cultures assayed at each time interval, and displayed in relation to endogenous Cdc6 mRNA (Northern blot analysis; calculated as multiples of the hybridization signal at  $t = 0$  hr) and to the fraction of cells in S phase (flow cytometry). Similar results were obtained in each of four separate transfection assays. A schematic representation of the human Cdc6 promoter illustrates E2F binding sites in relation to the transcriptional start site (arrow) of the human Cdc6 gene (14), and disruption of these sites (X) in the mutated promoter.

expression of the endogenous Cdc6 gene, and concomitantly activated the exogenous reporter gene controlled by the human Cdc6 promoter.

Nucleotide substitutions within two E2F binding motifs within the Cdc6 promoter (14) disrupted recognition of these sites by E2F proteins, as assessed by electrophoretic mobility shift assays (not shown) by using probes that represented native or mutated Cdc6 promoter sequences. As assessed by transient transfection assays in 3T3 cells, the human Cdc6 promoter bearing mutations in both E2F sites was actively transcribed under serum starved conditions in which the endogenous murine Cdc6 gene and the wild-type human Cdc6 promoter were transcriptionally inactive (Fig. 4B). These data reveal a role for E2F in the control of Cdc6 transcription and suggest that the Cdc6 gene is repressed in quiescent cells by an E2F complex, in a manner similar to certain other downstream targets of the E2F pathway, including E2F1 (23), B-Myb (24), and Orc1 (25). The observation that overexpression of E2F proteins led to activation of the endogenous Cdc6 gene could represent displacement or inactivation of an E2F repressor complex.

**Immunodepletion of Cdc6 in Human Cells.** To determine whether human Cdc6 is required for initiation of DNA replication, an affinity-purified, polyclonal anti-Cdc6 antibody was microinjected into HeLa cells. On the premise that immunodepletion would be most effective if Cdc6 were neutralized prior to formation of prereplication complexes, control or anti-Cdc6 antibodies were introduced into metaphase cells, and the ability of daughter cells to initiate DNA replication in the next cell cycle was assessed by the incorporation of BrdUrd. Cells injected at metaphase with either antibody were able to complete mitosis and cytokinesis, and microinjected antibodies could be detected in both daughter cells for at least 18 hr.

The majority of cells receiving the anti-Cdc6 antibody failed to initiate DNA replication (Fig. 5A-C). Only a small proportion (11%) of cells receiving anti-Cdc6 antibody remained competent to initiate DNA replication, and these cells exhibited less intense immunofluorescent staining of the microinjected rabbit IgG, suggesting that the quantity of anti-Cdc6 antibody in these cells may have been insufficient to titrate out endogenous Cdc6. In contrast, cells receiving control antibodies (purified nonimmune IgG, Zymed) initiated DNA replication during a subsequent 18 hr incubation time (Fig. 5D) at a frequency indistinguishable from that observed in uninjected cells (80–90%). A quantitative summary of the microinjection data is shown in Fig. 6.

## DISCUSSION

Three major conclusions are supported by this work. First, expression of mammalian Cdc6 is limited to proliferating cells. This is true for human diploid fibroblasts and human T24 epithelial cells propagated in culture, and for mucosal cells of the mouse duodenum growing within the intact animal. Second, transcription of mammalian Cdc6 genes is regulated by E2F transcription factors. This conclusion is based on transactivation of the endogenous human Cdc6 promoter under conditions of growth arrest in murine 3T3 cells. Third, Cdc6 is required for DNA replication in mammalian cells. The time course of Cdc6 expression in advance of the G<sub>1</sub>/S transition in human cells released from contact inhibition is consistent with a role for Cdc6 in establishing prereplication complexes, and immunodepletion of Cdc6 prevents initiation of DNA replication in HeLa cells.

The manner in which the activity of E2F transcription factors is modulated by interactions with members of the retinoblastoma protein (pRb) family, under the control of cyclin dependent protein kinases, has been characterized extensively in mammalian cells. Inherited or acquired genetic abnormalities that perturb this pathway are a common feature



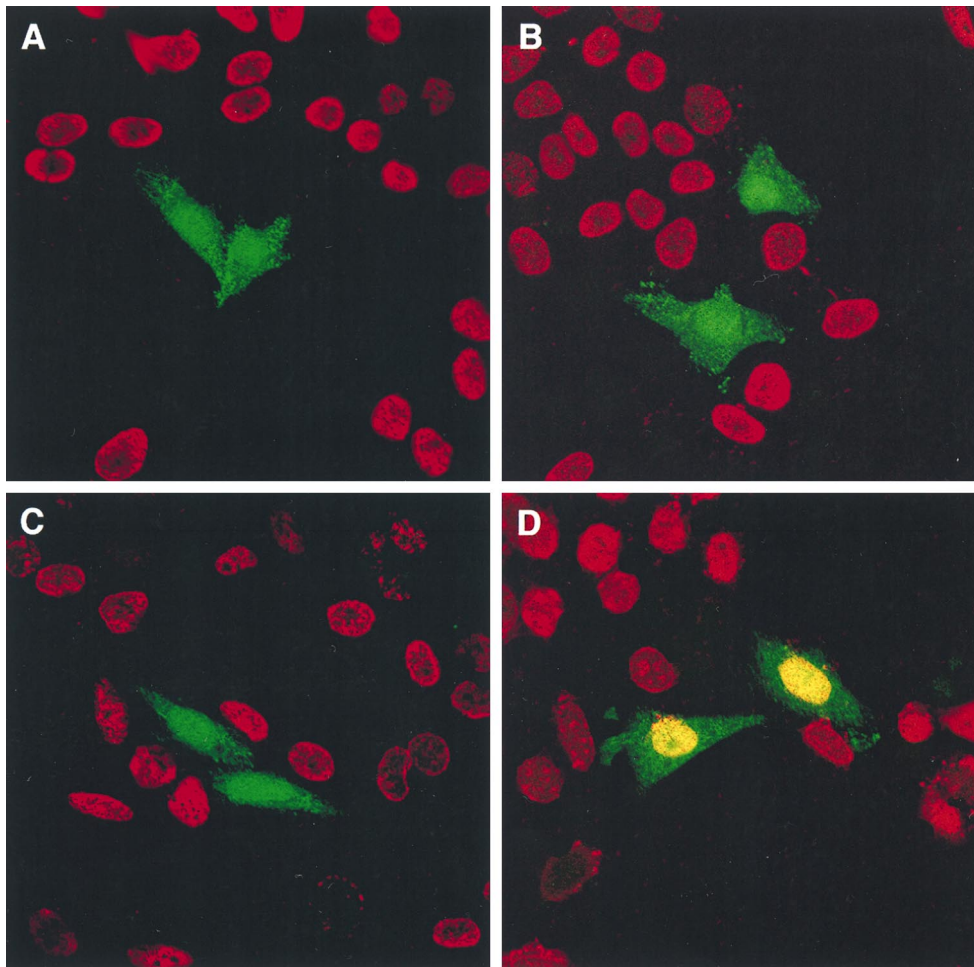


FIG. 5. Immunodepletion of Cdc6 blocks DNA replication in HeLa cells. Metaphase cells were microinjected with affinity purified anti-Cdc6 (A–C) or control non-immune IgG (D), each at a concentration of  $1.5 \mu\text{g}/\mu\text{l}$ , and cultured subsequently for 18 hr in the presence of bromodeoxyuridine (BrdUrd). Microinjected antibodies (green) and nuclear incorporation of BrdUrd (red) were detected by immunofluorescence microscopy. The nuclei of microinjected cells that incorporate BrdUrd appear yellow in the double immunofluorescence images.

of human malignancies (22, 26), and viral oncoproteins transform human cells, at least in part, through interactions that lead to deregulation of E2F activity (27).

By comparison, the protein–protein and protein–DNA interactions that function directly to specify and control origins

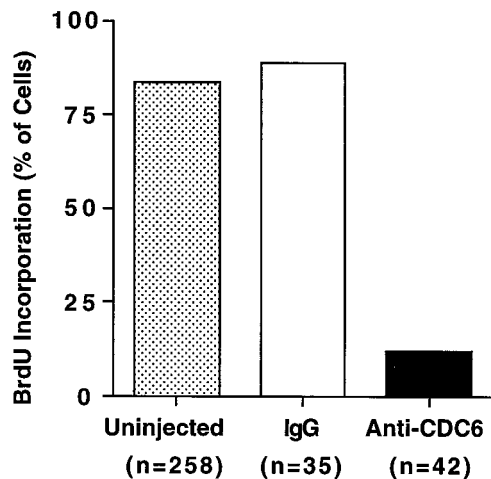


FIG. 6. Quantitative analysis of BrdUrd incorporation in uninjected cells and in cells microinjected with the indicated antibodies. The number of cells scored for DNA replication in each test condition is indicated.

of DNA replication in human cells are poorly defined, and current mechanistic understanding of replication initiation is based almost entirely on results from yeast models (1). The recent identification of human ORC (28) and Cdc6 (14) proteins provides new opportunities to advance knowledge in this area. Previous work has shown that overexpression of E2F proteins can lead to an induction of S phase in otherwise growth-arrested cells (29–32), indicating that downstream targets of E2F are capable of promoting initiation of DNA replication. While our current results do not define molecular details of the mechanisms by which Cdc6 transcription is regulated by E2Fs, our data are consistent with the previous observation that human ORC1 transcription is controlled by E2F (25) and link the pRb–E2F pathway to the control of replication origins in human cells. What remains to be determined is the extent to which E2F imparts only negative control of the Cdc6 gene or whether E2F also contributes directly to positive control as cells reenter the cell cycle. For instance, it is possible that overexpression of E2F activity in quiescent cells inactivates or displaces an endogenous E2F repressor complex, thus leading to activation of the gene. Further experiments will be necessary to ascertain whether direct activation of Cdc6 transcription by E2F complexes also contributes to physiological regulation of DNA replication.

Cdc6 gene expression in rapidly proliferating *S. cerevisiae* is under transcriptional control during late G<sub>2</sub>, and yeast Cdc6 is expressed most abundantly in the period between late M phase and early S phase (6, 33). Under conditions in which G<sub>1</sub> is

prolonged, a second peak of yeast Cdc6 gene transcription in late G<sub>1</sub> is driven by factors that are functionally analogous to mammalian E2F proteins with respect to control by cyclin-dependent kinases and mitogenic signaling pathways (3, 7). Other mechanisms that govern the abundance of Cdc6 in yeast, however, may differ from those responsible for its regulation in mammalian cells. Yeast Cdc6 is an unstable protein, and degradation of Cdc6 following S phase contributes to its prominent cyclic variation across the yeast cell cycle (6). Our current analysis of Cdc6 expression in asynchronously or synchronously replicating human T24 cells, like our previous studies of HeLa cells separated by centrifugal elutriation (14), did not reveal evidence for a major decline in the abundance of Cdc6 following S phase in cycling cells, but additional experiments are required to provide a definitive understanding of the cell cycle dependent regulation of Cdc6 in human cells, as opposed to the growth regulated control examined in these current studies.

In general, the data presented here support the view that fundamental molecular mechanisms controlling initiation of DNA replication are conserved in all eukaryotes. We cannot yet ascertain the extent to which human Cdc6 manifests the full repertoire of distinctive and interesting properties of its yeast homologs. The finding that Cdc6 is required for initiation of DNA replication in human cells suggests, however, that deregulated expression of Cdc6 may play a role in oncogenic transformation or disease progression in human cancers, and that Cdc6 provides a good target for biotechnological strategies designed to arrest cell proliferation.

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