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Human Cyclin E, a Nuclear Protein Essential for the G_1-to-S Phase Transition

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Cyclin E was first identified by screening human cDNA libraries for genes that would complement G_1 cyclin mutations in *Saccharomyces cerevisiae* and has subsequently been found to have specific biochemical and physiological properties that are consistent with it performing a G_1 function in mammalian cells. Most significantly, the cyclin E-Cdk2 complex is maximally active at the G_1/S transition, and overexpression of cyclin E decreases the time it takes the cell to complete G_1 and enter S phase. We have now found that mammalian cells express two forms of cyclin E protein which differ from each other by the presence or absence of a 15-amino-acid amino-terminal domain. These proteins are encoded by alternatively spliced mRNAs and are localized to the nucleus during late G_1 and early S phase. Fibroblasts engineered to constitutively overexpress either form of cyclin E showed elevated cyclin E-dependent kinase activity and a shortened G_1 phase of the cell cycle. The overexpressed cyclin E protein was detected in the nucleus during all cell cycle phases, including G_0. Although the cyclin E protein could be overexpressed in quiescent cells, the cyclin E-Cdk2 complex was inactive. It was not activated until 6 to 8 h after readdition of serum, 4 h earlier than the endogenous cyclin E-Cdk2. This premature activation of cyclin E-Cdk2 was consistent with the extent of G_1 shortening caused by cyclin E overexpression. Microinjection of affinity-purified anti-cyclin E antibodies during G_1 inhibited entry into S phase, whereas microinjection performed near the G_1/S transition was ineffective. These results demonstrate that cyclin E is necessary for entry into S phase. Moreover, we found that cyclin E, in contrast to cyclin D1, was required for the G_1/S transition even in cells lacking retinoblastoma protein function. Therefore, cyclins E and D1 control two different transitions within the human cell cycle.

Cyclins and their catalytic subunits, the cyclin-dependent kinases (Cdks), play key roles in the regulation of eukaryotic cell cycle events (see references 10 and 82 for reviews). Cyclins are believed to determine the subcellular localization, substrate specificity, interaction with upstream regulatory proteins, and timing of activation of Cdks.

Cell cycle control mechanisms have been highly conserved throughout evolution (see reference 59 for a review). Cyclins were originally discovered in marine invertebrates. In clam and sea urchin embryos, A- and B-type cyclins were found to accumulate to high levels during interphase before they were abruptly degraded during mitosis (17). Control of the cell cycle by these cyclins was demonstrated when injection of clam cyclin A mRNA was able to advance G_2-arrested *Xenopus* oocytes into meiotic metaphase (89). Furthermore, after destruction of all mRNA in *Xenopus* egg extracts, readdition of sea urchin cyclin B message was sufficient to drive the extract into mitosis (56).

In mammalian cells, several cyclins have been identified and classified according to their sequence similarities. The first cyclins to be identified in humans were the A (69, 97) and B (68) types, which are essential for progression through S, G_2, and M. By analogy with cell cycle regulation in the yeast *Saccharomyces cerevisiae*, it was anticipated that human cells would also contain specific cyclins necessary for progression through the G_1 phase of the cell cycle. In fact, several new cyclins were then identified by screening human cDNA libraries for genes that could complement G_1 cyclin mutations in *S. cerevisiae*: cyclin C, cyclin D1, and cyclin E (41, 44, 101). The cyclin D1 gene was independently discovered as the gene located at a chromosome rearrangement in a parathyroid tumor (54), as a candidate *bcl-1* oncogene (99), and as a gene whose transcription is induced in early G_1 in response to extracellular mitogens (51). However, the A- and B-type cyclins were also found to rescue G_1 cyclin mutations, albeit with a lower efficiency than cyclin E (41, 44). Thus, additional, more direct evidence was needed to support the idea that cyclin D1, C, or E functioned during G_1 in mammalian cells.

Since then, it has been shown that cyclin D1 is both necessary and sufficient to control progression through at least part of the G_1 phase in mammalian cells (2, 72). To carry out its function, cyclin D1 associates with Cdk4 (92), its catalytic subunit (49, 103). The kinase activity of the cyclin D1-Cdk4 complex is maximal in early to mid-G_1 phase (50), and it is thought that this kinase phosphorylates and inactivates the retinoblastoma protein (pRb) during G_1 (19, 38). Overexpression of cyclin D1 shortens the G_1 phase of the cell cycle (72, 73), and inhibition of cyclin D1 function prevents entry into S phase (2, 72).

Cyclin E associates with Cdk2 and activates its serine-threonine kinase activity shortly before entry into S phase (14, 42). Overexpression of cyclin E decreases cell size, diminishes the requirement for growth factors, and accelerates the G_1 phase of the cell cycle (61, 73, 98). In addition, cyclin E-Cdk2 complex has been shown to be a target for regulators of the G_1 progression such as p21 (also called Cip1, Pic1, Sdi1, and Waf1) and p27 (also called Ick, Kip1, and Pic2) (see references 16 and 67 for reviews). These data suggest that cyclin E is also an essential element for controlling G_1 progression in mam-

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malian cells. In this paper we have focused on the function of human cyclin E in the regulation of the G_1/S transition.

MATERIALS AND METHODS

Immunoreagents. The preparation, purification, and characterization of the rabbit polyclonal antibody to human cyclin E (41, 42) and the mouse monoclonal antibody to human cyclin D1, clone DCS-6 (47), have been described previously. The rabbit polyclonal antisera to human cyclin A (48), Cdk2 (42, 63), and Cdc2 (42) and the monoclonal antibodies to human cyclin E (20) and p21 (15) were described previously. Antibromodeoxyuridine (anti-BrdU) monoclonal antibody-nuclease, ready to use, was purchased from Amersham Inc., and anti-BrdU sheep polyclonal antibody was purchased from Fitzgerald Inc.

Cloning of cyclin E-L. RNA from proliferating HL-60, HeLa, and Manca cells was reverse transcribed by random priming and directionally cloned into lambda phage vectors. Phage hybridizing to a probe derived from the 5′ end of the cyclin E cDNA HU4 (42) were further purified, and the cDNAs that they contained were sequenced.

RT-PCR. Reverse transcriptase PCR (RT-PCR) was carried out using one primer which hybridizes to cyclin E exon 1 (5′ CGCCTGCCGGGACTGGAG 3′) and one primer which hybridizes to cyclin E exon 3 (5′ TCTTCCTGGAGC GAGCCG 3′).

Construction of retrovirus vectors and virus infection. The full-length cDNA coding human cyclin E-S (1.6-kb *Hin*dIII fragment) or cyclin E-L (1.7-kb *Hin*dIII fragment) was treated with Klenow fragment and cloned into the *Hpa*I site of retrovirus vectors LXSN (G418 resistant) and LXSH (hygromycin resistant). Preparation of recombinant retroviruses and infection of Rat-1 and human fibroblasts with these expression vectors was performed as described elsewhere (52).

Immunoblotting and immunoprecipitation. Cell lysates for histone H1 kinase assay were prepared by lysing cells (10^6) in 250 μl of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40) containing 50 mM NaF, 0.1 mM sodium orthovanadate, and 10 μg (each) of aprotinin, leupeptin, and pepstatin A per ml. Total-cell extracts were prepared by directly suspending cells in Laemmli 2× sodium dodecyl sulfate (SDS) sample buffer. Immunoprecipitation and histone H1 kinase assay were performed with specific antibodies against cyclin A or E as described before (42). For metabolic labeling with [^{35}S]methionine, confluent cultures (in 10-cm-diameter dishes) were washed twice with methionine-free, cysteine-free Dulbecco's modified Eagle's medium (DMEM) (ICN), and then the same medium containing 10% dialyzed calf serum and Trans^{35}S-label (ICN) (150 μCi/ml) was added and incubated for 6 h before cell lysis. Cells were washed twice with cold phosphate-buffered saline (PBS) and directly lysed in 1 ml of lysis buffer containing 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 10 μg of pepstatin A per ml, 50 mM NaF, and 0.1 mM sodium orthovanadate. Cells were scraped and transferred to an Eppendorf tube and further incubated for 10 min on ice. Lysates were prepared by centrifugation at 15,000 × *g* for 10 min and then precleared by incubation with 100 μl of protein A-Sepharose (50%, vol/vol) for 30 min followed by centrifugation at 15,000 × *g* for 5 min. Two microliters of affinity-purified cyclin E antibody was added to the lysate (250 μl) and incubated for 3 h on ice, and then 25 μl of protein A-Sepharose (50%, vol/vol) was added and incubated for 30 min at 4°C with a rotator. After brief centrifugation at 15,000 × *g*, immunoprecipitates were washed three times with lysis buffer and resuspended in 2× SDS sample buffer. For metabolic labeling of the stimulated cells (16 h), a 50% confluent synchronized culture (10-cm-diameter dish) was washed at 13 h after serum stimulation with the labeling medium and labeled for 3 h as described above. Unstimulated cells (0 h) were also labeled for the last 3 h of synchronization as described above, in the absence of dialyzed serum.

Immunofluorescence. Cells growing on glass coverslips were washed twice with PBS, fixed with PBS containing 3.7% formaldehyde for 10 min at room temperature, and permeabilized with 0.5% Nonidet P-40 in PBS for 5 min at room temperature. After washing with PBS, cells were incubated for 1 h at room temperature with affinity-purified cyclin A and cyclin E antibodies (cyclin A, diluted 1:500, 0.4 μg/ml; cyclin E, diluted 1:200, 1 μg/ml; dilutions in PBS plus 10% goat serum). They were then washed with PBS and incubated with Texas red-conjugated goat anti-rabbit immunoglobulin G antibodies (dilution 1:100 in PBS; Cappel). After washing with PBS, coverslips were mounted with 90% glycerol–10% PBS–3% *n*-propyl-galate. Fluorescence and phase-contrast images were obtained with Bio-Rad MRC-600 attached to a Nikon microscope. Essentially the same pattern of nuclear staining with affinity-purified cyclin E antibodies was obtained with alcohol fixation (cold methanol, 3 min at −20°C) (not shown).

Cell culture and cell synchronization. Human lung fibroblasts IMR-90 were obtained from the American Type Culture Collection at population doubling 20 and grown as described previously (91) for not more than 40 total population doublings. Cells were counted at each passage, and the increase in population doubling was calculated as the $\log_2$ fold increase in cell number. IMR-90+LT cells (simian virus 40 [SV40] large-T-transfected IMR-90 cells) were provided by J. W. Shay and grown as described elsewhere (92). Human diploid foreskin fibroblasts (NHF cells; provided by T. Norwood at passage six), human breast adenocarcinoma cell lines MCF-7 and MDA-MB-468, human fibrosarcoma cell line HT-1080, human epitheloid cervical carcinoma cell line HeLa, WI-38-VA-13 cells (SV40 large-T-transfected lung fibroblasts), and Rat-1 fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U of penicillin per ml, and 1 μg of streptomycin per ml.

Different cell types were synchronized in G_0 or G_1 according to the description in reference 60. Briefly, NHF cells, NHF cells expressing cyclin E-L and cyclin E-S, and IMR-90 cells were incubated for 2 to 3 days in DMEM containing 0.2% FCS. After this period, less than 1% of the cell population incorporated BrdU during an incubation of 24 h, and flow cytometry confirmed that greater than 95% of the cells had a 2N (G_0/G_1) DNA content. IMR-90+LT cells contained a dexamethasone-inducible SV40 large T antigen gene and were incubated for 2 days in the presence of 10% steroid-depleted FCS (obtained as described previously [100]) and for 3 additional days in the presence of 0.2% steroid-depleted FCS. After these periods of incubation, less than 8% of the cell population (cells that are still large T positive) incorporated BrdU during an incubation of 24 h, as detected by immunofluorescence (64). This result correlated with cell cycle analysis by flow cytometry, demonstrating that in these cultures more than 95% of the cells presented a 2N DNA content (81). In the experiments in which IMR-90 and IMR-90+LT cells were directly compared, both cell types were synchronized as described for IMR-90+LT. Tumor cell lines were synchronized in mitosis by an 18-h incubation in the presence of 40 ng of nocodazole (Sigma) per ml. The rounded cells were subsequently detached from the substrate by gentle shaking and pipetting. After two washes in fresh DMEM (nocodazole free), the cells were replated into fresh medium. After 4 h, the nonattached cells were washed out. Flow cytometry demonstrated that in these cultures more than 95% of the cells presented a 2N DNA content until about 12 to 14 h after replating.

Cell cycle phases were monitored by flow cytometry (FACScan; Becton Dickinson) after staining cellular DNA with 50 μg of propidium iodide (Sigma) per ml in the presence of 10 μg of DNase-free RNase (Boehringer Mannheim) per ml, by BrdU incorporation, and by incorporation of [^{3}H]thymidine into the acid-insoluble fraction of the cell lysates (42).

Microinjection. Cell monolayers growing on glass coverslips (at ca. 60% density) were synchronized in G_0 or G_1 as above described and microinjected with the indicated antibodies by an automated microinjection system (Zeiss) (1). All microinjection experiments were carried out in 3.5-cm-diameter petri dishes containing 3 ml of carbonate-free DMEM, in order to avoid a decrease in the pH of the medium during the injection. Each cell was injected with antibodies (2.5 mg/ml in PBS) at a pressure between 50 and 150 hPa. The computer settings for injection were as follows angle, 45°; speed, 10; and time of injection, 0.0 s. For more details on the microinjection procedure, see reference 65.

DNA synthesis was monitored by incubation with BrdU (100 μM; Amersham) for different times (see Results) before fixing of the cells. Coverslips were rinsed in PBS, fixed for 10 min in −20°C cold methanol-acetone (1:1), and washed again three times with PBS. Cells were incubated for 1 h with biotinylated horse anti-rabbit antibody or with biotinylated horse anti-mouse secondary antibody (Vector Laboratories; dilution, 1:50), washed three times with PBS, and incubated with Texas red-conjugated streptavidin (Vector Laboratories; dilution, 1:100) or fluorescein isothiocyanate-conjugated streptavidin (Vector Laboratories; dilution, 1:50). Coverslips were subsequently incubated for 10 min with 1.5 M HCl, washed three times with PBS, then incubated for 1 h with a solution of mouse monoclonal anti-BrdU antibody plus nuclease (undiluted; Amersham), and then incubated for 30 min with a 1:50 dilution of an anti-mouse fluorescein isothiocyanate-conjugated antibody (Vector Laboratories). In double labeling experiments, if the first antigen was detected with an anti-mouse antibody, we stained BrdU with a sheep polyclonal antibody (Fitzgerald; dilution, 1:50) followed by an anti-sheep fluorescein isothiocyanate-conjugated antibody (Vector Laboratories; dilution, 1:50) for 30 min or directly with a fluorescein isothiocyanate-conjugated mouse monoclonal anti-BrdU antibody (undiluted; Becton Dickinson).

All antibody reactions were carried out in a humidified chamber at room temperature, and dilutions were made in DMEM containing 10% FCS. Counterstaining for DNA was performed by adding 1 μg of bisbenzimide (Hoechst 33258) per ml into the final PBS wash. Immunofluorescence samples were directly mounted in Crystal/mount medium (Biomeda Corp.). Photographs were taken with a Plan-Neofluar 40 X lens mounted on a Zeiss Axiovert Photomicroscope and a Color Video Printer Mavigraph on Sony UPC-3010 print paper.

In each experiment about 100 injected cells (and a corresponding number of noninjected cells) were counted. The percent inhibition of BrdU incorporation was calculated as $[(N - I)/N] \times 100$, where N is the percentage of BrdU incorporation in noninjected cells and I is the percentage of BrdU in cells microinjected with antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU-positive cells that had not been injected.

RESULTS

Two human cyclin E proteins encoded by alternatively spliced mRNAs. Three classes of cyclin E cDNAs containing different sequence arrangements at their 5′ ends were isolated from libraries of Manca, HeLa, and HL-60 cell cDNAs (Fig.

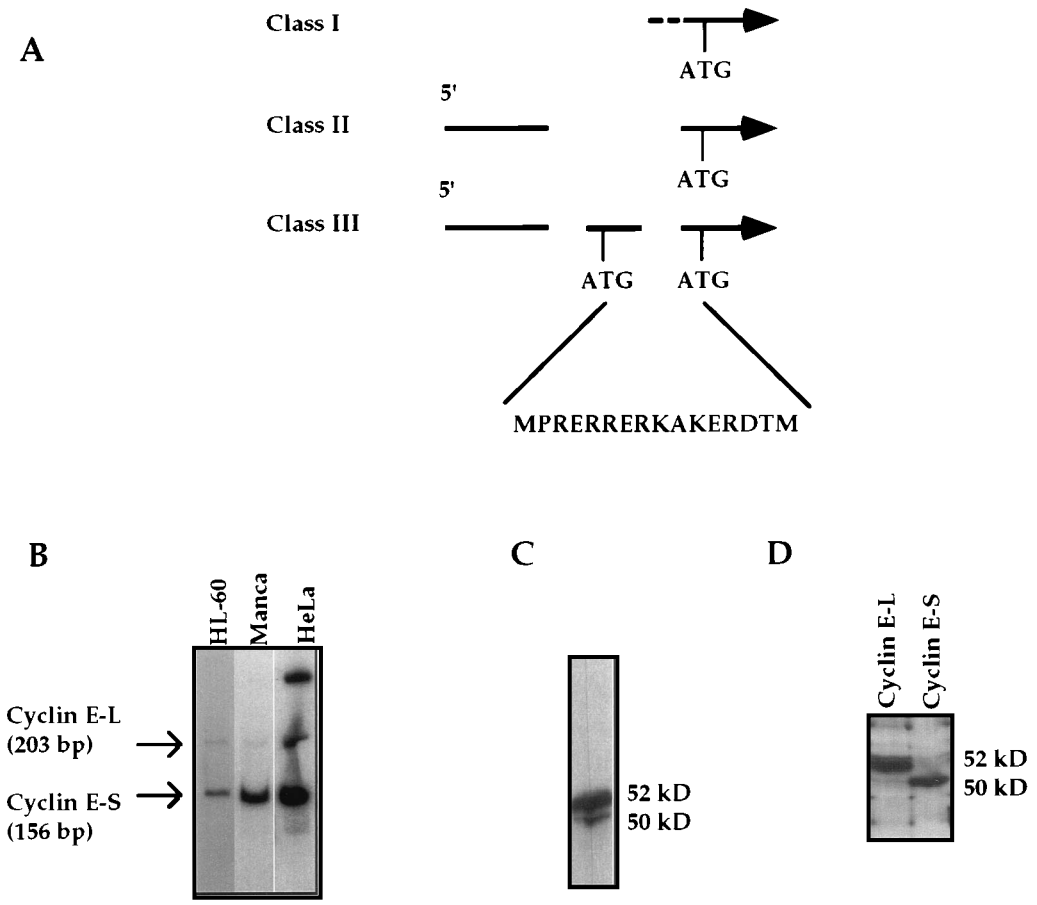

FIG. 1. Alternatively spliced human cyclin E mRNAs encode different forms of the cyclin E protein. (A) Schematic representation of two alternatively spliced forms of cyclin E mRNA detected in human cells. Shown are the first three exons of cyclin E-L; cyclin E-S lacks the second exon. Translation of cyclin E-L is initiated at an ATG codon located in exon 2. This ATG is in frame with the initiating ATG codon for cyclin E-S in exon 3. The peptide sequence for the amino-terminal portion specific to cyclin E-L is shown. (B) Alternatively spliced cyclin E mRNAs in vivo. Total cellular RNA from the indicated cell types was reverse transcribed with random hexamers, and the reverse transcription products were amplified by PCR using a cyclin E-specific primer located in exons 1 and 3 in the presence of [^{32}P]dCTP. Indicated are two RT-PCR products. On the basis of cDNA sequences, they are the size expected from alternatively spliced cyclin E mRNAs. Primer pairs from exons 3 and 5 generate a single RT-PCR product of the expected size from the same RNA samples (not shown). (C) Expression of the cyclin E-L and cyclin E-S proteins. Affinity-purified anti-cyclin E polyclonal antibodies were used to detect the cyclin E protein by immunoblotting extracts from peripheral blood lymphocytes induced to proliferate in vitro. The 52- and 50-kDa proteins are of the molecular sizes predicted for cyclin E-L and cyclin E-S, respectively. (D) cDNAs corresponding to cyclin E-L and cyclin E-S were expressed in the murine NIH 3T3 cell line with retrovirus expression vectors. As predicted, the vector encoding cyclin E-L specifically overproduced a 52-kDa protein, and the vector encoding cyclin E-S specifically overproduced a 50-kDa protein.

1A). The first class (one isolate) was identical to a cyclin E cDNA previously recovered from a human glioblastoma cDNA library during a screen for genes which could complement mutations in *S. cerevisiae* G_1 cyclins (7, 41). The second class (one isolate) differed in the 5′ untranslated region but encoded a protein identical to the first. The third type of cDNA (four isolates) was identical to the second except that it contained a 47-bp insertion beginning at nucleotide 140. As a consequence, this cDNA encodes a protein that is 15 amino acids longer at its amino terminus than the protein encoded by the class I and II cDNAs. The cyclin E protein encoded by the class III cDNA is called cyclin E-L, and the smaller protein encoded by the class I and II cDNAs is called cyclin E-S.

In order to determine the origin of these cDNAs, we mapped the human cyclin E gene to chromosome 19q12-13 (9) and obtained cosmid clones containing the cyclin E gene from a chromosome 19 genomic library (H. Mohrenweiser, Lawrence Livermore National Laboratory). Cosmid 9924 was subcloned, and a 2.0-kb *Sac*I fragment containing the 5′ end of the cyclin E gene was sequenced. Comparison of this genomic DNA sequence with the sequence of the cyclin E cDNAs revealed that the class II and III cDNAs derive from alternatively spliced transcripts which differ by the presence or absence of a single exon (designated exon 2). Exon 1 is untranslated, and exon 2 contains a start methionine which is in frame with the first ATG codon in exon 3 (the start methionine used in the class I and II cDNAs). Therefore, the class III cDNA (which contains exon 2) encodes a protein, cyclin E-L, which is 15 amino acids longer at its amino terminus but otherwise identical to cyclin E-S. Two cyclin E cDNAs encoding proteins with different amino termini have also been identified in *Drosophila melanogaster* and shown to be the products of an alternatively spliced primary transcript (75). However, the amino-terminal addition in *Drosophila* cyclin E has no significant homology to the amino-terminal addition found in mammalian cyclin E-L. The origin of the class I cDNA is uncertain, but it appears to have been generated from an incompletely processed transcript since its 5′ end is at a *Not*I site located within the intron between exons 2 and 3. Thus, the independent isolation of this cDNA from two different libraries is because

*Not*I cleavage was used during preparation of both cDNA libraries.

Total cellular RNA isolated from three cell types was analyzed by RT-PCR in order to demonstrate directly the presence of class II and III cyclin E transcripts in cellular RNA (Fig. 1B). Primers for PCR were homologous to sequences in exons 1 and 3. Thus, the PCR products were specific for class II and III cDNAs and differed in size by 47 bp (the length of exon 2). The predicted PCR products were observed with RNA from each cell type. Consistent with the presence of two cyclin E mRNAs, immunoblotting of whole-cell lysates with affinity-purified anti-cyclin E antiserum revealed two species of cyclin E protein with apparent molecular masses of approximately 50 and 52 kDa (Fig. 1C). In all the cell types that we examined, the 52-kDa cyclin E protein was always more abundant than the 50-kDa form. To test whether the 50- and 52-kDa forms of cyclin E correspond to cyclin E-S and cyclin E-L, respectively, retroviral vectors containing either the class II or the class III cDNA were constructed. Murine fibroblasts infected with the retrovirus containing the class II cDNA overexpressed a protein which comigrated with the 50-kDa form of endogenous cyclin E, and murine fibroblasts infected with the retroviral vector containing the class III cDNA overexpressed a protein comigrating with the 52-kDa form of endogenous cyclin E (Fig. 1D). These data support the conclusion that cyclin E-L is the major form of the cyclin E protein in mammalian cells and that cyclin E-S protein is also expressed in vivo but at a lower level. Mammalian cyclin E has also been reported to exist in a third isoform, which is thought to result from phosphorylation and correlate with activation of the cyclin E-Cdk2 complex (12). This form of cyclin E has a reduced electrophoretic mobility compared with that of cyclin E-L. We observed this form of cyclin E in cyclin E immunoprecipitates from extracts of proliferating cells overexpressing cyclin E-L but not in parallel immunoprecipitates from quiescent cells (where the cyclin E-associated kinase is inactive) (see Fig. 6). Nevertheless, we cannot completely exclude the possibility that in proliferating cells a portion of the 52-kDa form of cyclin E derives from phosphorylation of the 50-kDa cyclin E-S.

Characterization of cyclin E-L. Overexpression of cyclin E-S accelerates the G_1 phase of the cell cycle, decreases cell size, and diminishes the serum growth factor requirement for cell proliferation (61, 73, 98). All of these properties are consistent with the idea that the abundance of the cyclin E protein determines that rate at which cells progress through G_1. However, since cyclin E-L appears to be the major form of cyclin E expressed in vivo, it was of interest to determine whether cyclin E-L had similar functional properties. Rat-1 fibroblasts were infected either with control virus or with the cyclin E-L cDNA-containing virus. For these experiments pools of more than 1,000 infected cells were studied, thereby eliminating the possibility that our results were due to clonal heterogeneity commonly observed in cultured cells. Infection with the cyclin E-L cDNA-containing retrovirus increased the level of the 52-kDa form of cyclin E about fivefold (Fig. 2A). Flow cytometric analysis of exponentially proliferating cells demonstrated that overexpression of cyclin E-L decreased the percentage of cells in the G_1 phase of the cell cycle (Fig. 2B) and decreased cell size (not shown) compared with that of control cells. The magnitude of these effects was at least as great as that observed in cells overexpressing cyclin E-S (61). We also constructed cells which expressed still higher levels of cyclin E-L by double infection by using two retroviral vectors containing either the G-418 or hygromycin resistance marker. In this pool of doubly infected cells cyclin E-L was expressed at a level about 10-fold higher than that in control cells (Fig. 2A), and the percentage

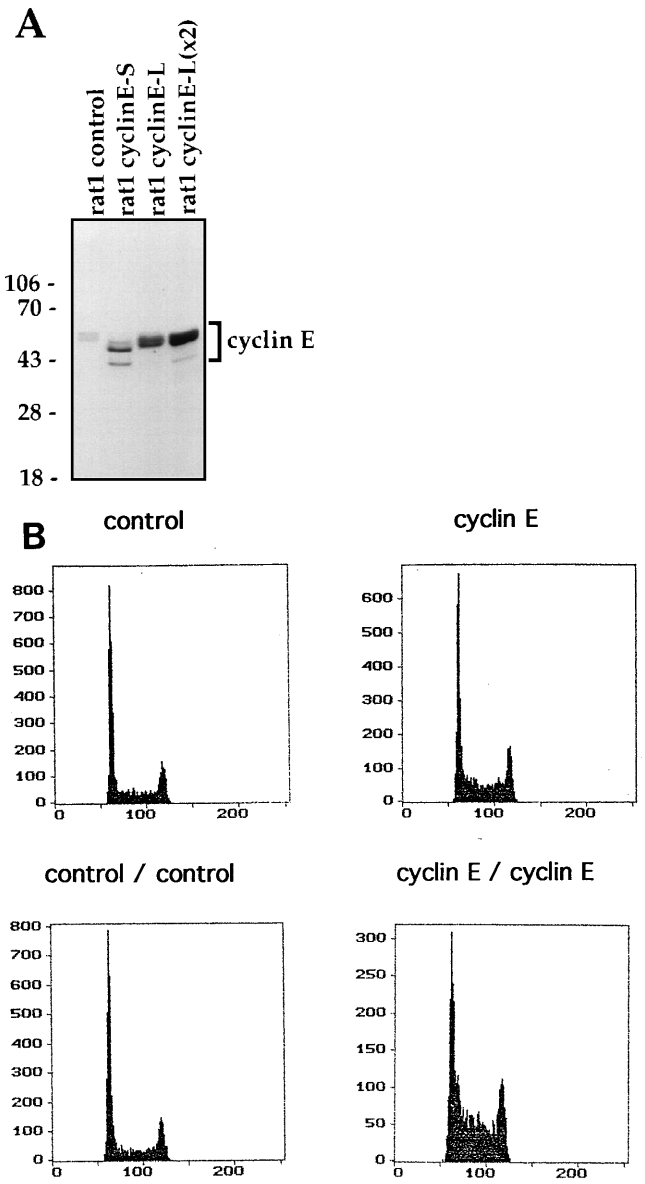

FIG. 2. Enforced expression of cyclin E-L in Rat-1 fibroblasts by using retrovirus vectors. (A) Immunoblot analysis. Expression of cyclin E was determined in total-cell extracts from exponentially growing cells (2×10^5 cells per lane) by immunoblotting with affinity-purified antibodies to cyclin E following SDS-PAGE. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham). Positions of two (50- and 52-kDa) cyclin E proteins are indicated. Molecular size markers are indicated on the left (in kilodaltons). Lanes: rat1 control, Rat-1 fibroblast infected with vector virus; rat1 cyclinE-S, Rat-1 fibroblast infected with cyclin E-S cDNA-containing retrovirus; rat1 cyclinE-L, Rat-1 fibroblast infected with cyclin E-L cDNA-containing retrovirus; rat1 cyclinE-L(x2), Rat-1 fibroblast doubly infected with the cyclin E-L cDNA-containing retroviruses. (B) Effect of double infection of cyclin E-L cDNA-containing retrovirus on the cell cycle of Rat-1 fibroblasts. Rat-1 fibroblasts infected with LXSN (control), LXSN and LXSH (control/control), LXSN–cyclin E-L (cyclin E), and LXSN–cyclin E-L and LXSH–cyclin E-L (cyclin E/cyclin E) were plated at 5×10^5 cells per 100-mm-diameter dish with DMEM supplemented with 10% calf serum. Two days later, cells were collected by trypsinization, fixed, and stained with DNA-specific dye, propidium iodide. One million cells in propidium iodide solution (0.5 ml) were analyzed by FACScan using LYSYSII software (Becton Dickinson). Histograms show relative DNA content (*x* axis) and cell number (*y* axis).

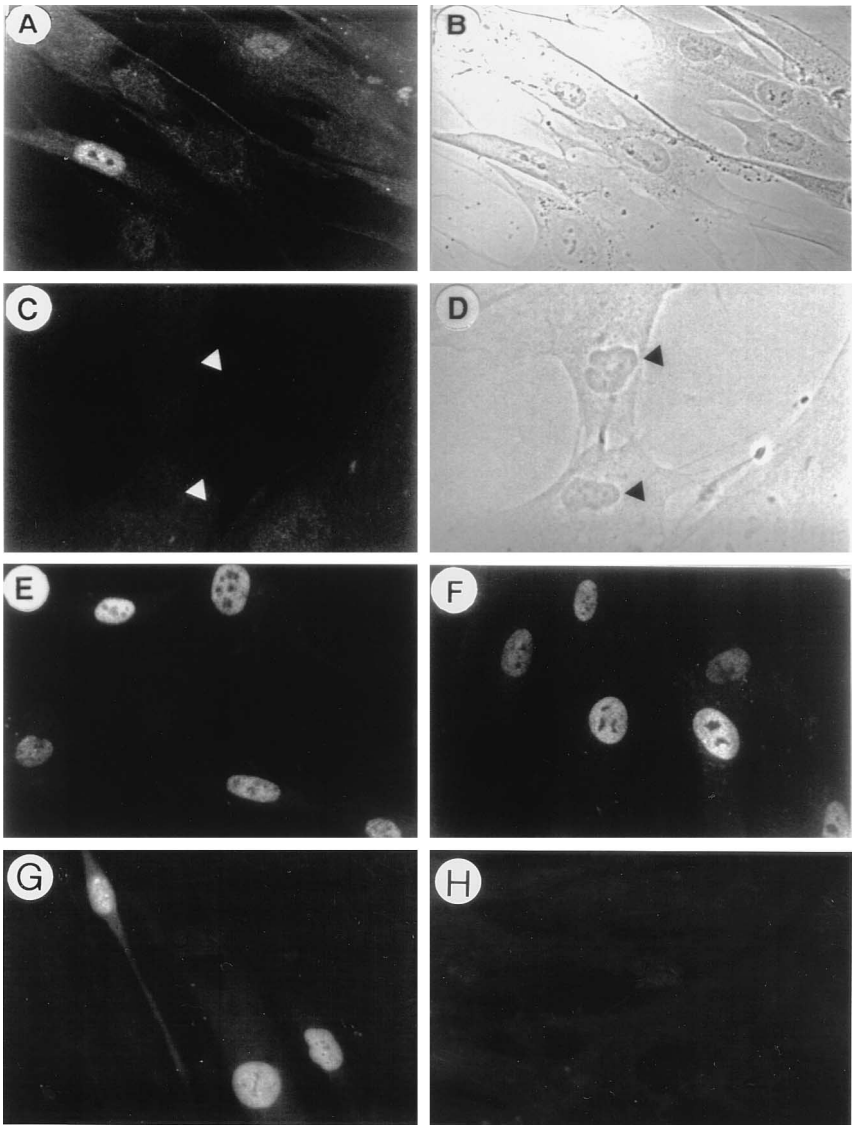

FIG. 3. Nuclear localization of cyclin E in normal human fibroblasts. (A and C) Asynchronous normal diploid human fibroblasts NHF cells, were fixed and stained with affinity-purified polyclonal anti-cyclin E antibodies; (B and D) phase-contrast images of the same field. (A and B) Population of asynchronous cells; (C and D) early-G_1 cells (marked with triangles), as evidenced by incomplete cytokinesis and partially condensed nuclear chromatin; (E and F) anti-cyclin E staining of exponentially growing cultures of NHF cells overexpressing cyclin E-S (E) or cyclin E-L (F); (G and H) asynchronous normal diploid human fibroblasts, IMR-90 cells, stained with anti-cyclin E monoclonal antibody with (H) or without (G) preincubation with purified bacterially expressed cyclin E.

of cells in G_1 was decreased to an even greater extent than in the singly infected cells (Fig. 2B). Thus, the degree of G_1 acceleration appeared to be related to the amount of cyclin E overexpression. The size of the doubly infected cells was not decreased significantly compared with that of the singly infected cells (not shown), and the population doubling time for both the singly and doubly infected cells was identical to that of the control (reference 61 and data not shown). Cyclin E-L and cyclin E-S both associate predominantly with Cdk2, and both are expressed in similar patterns during the cell cycle (not shown). We have also observed that cyclin E-L and cyclin E-S can both complement the triple cln1,2,3$^-$ deficiency in *S. cerevisiae* (not shown).

To determine the subcellular localization of cyclin E, exponentially growing normal human diploid fibroblasts were stained with affinity-purified anti-cyclin E antibodies. Staining was predominantly nuclear and markedly heterogeneous from cell to cell (Fig. 3A and B). Cyclin E showed a similar pattern of heterogeneous nuclear staining in other cell types, including Rat-1 fibroblasts, HeLa cells, HT-1080 fibrosarcoma cells, and SV40-transformed WI-38 human fibroblasts (not shown). Furthermore, the intensity of nuclear staining in each cell type correlated with the level of cyclin E expression found by immunoblotting. Four observations showed that the nuclear staining reflected detection of cyclin E. First, no nuclear staining was evident with either preimmune serum or anti-cyclin E antibody blocked by recombinant cyclin E protein (not shown). Second, cell lines infected by cyclin E-S or cyclin E-L cDNA-containing retroviruses exhibited a more intense and homogeneous nuclear staining consistent with the constitutive overexpression of cyclin E in these cells (Fig. 3E and F). Third, NIH 3T3 cells transiently transfected with a plasmid expressing a myc-epitope-tagged version of cyclin E-L showed exclusively nuclear staining when stained with an anti-myc epitope mono-

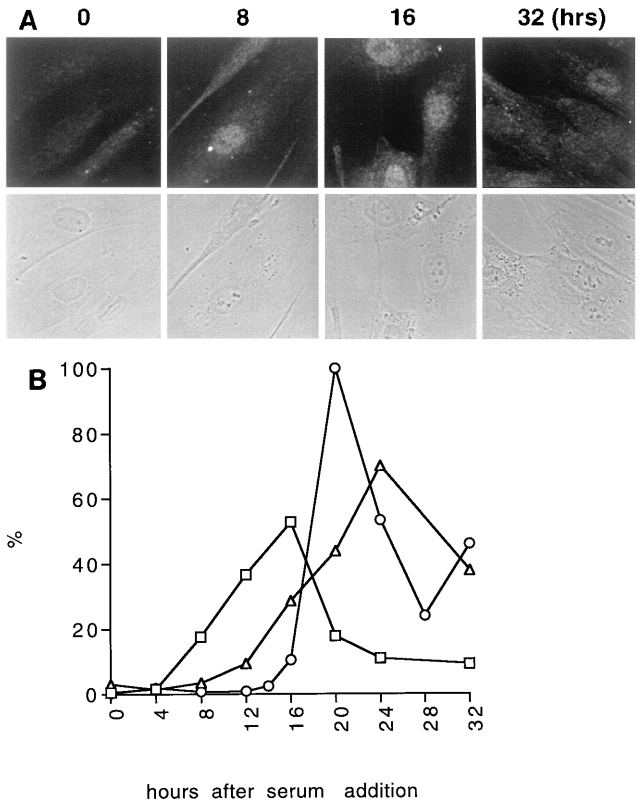

FIG. 4. Cell cycle-dependent expression of cyclin E in the nuclei of normal human fibroblasts. (A) Induction and localization of cyclin E after serum stimulation. G_0-arrested and restimulated normal human fibroblasts were fixed and stained with affinity-purified anti-cyclin E antibodies at 0, 8, 16, and 32 h after serum addition. Phase-contrast images of the same field are shown on the bottom. (B) Cyclin E expression precedes DNA synthesis. G_0-arrested cells were stimulated by the addition of serum, fixed, and stained to detect cyclin E (□) or cyclin A (△) at each time point with affinity-purified cyclin E or cyclin A antibody. Cells with nuclear staining for cyclin A or cyclin E under epifluorescence microscopy were counted. Each value represents the average from duplicate experiments with more than 300 cells. DNA synthesis in a duplicate culture was measured by incorporation of [^{3}H]thymidine into the acid-insoluble fraction (42). ○, relative amount of incorporated radioactivity. At least 50% of the cells in the culture were observed to enter S phase at 24 h after serum stimulation, on the basis of BrdU incorporation. The mitotic index of the cultures peaked at 28 h after stimulation, and a significant number of the cells entered the next G_1 phase at 32 h.

clonal antibody (39). Fourth, identical specific nuclear staining was also observed with a monoclonal antibody to cyclin E (Fig. 3G and H).

Previous characterization of cyclin E expression in synchronized cell populations showed that the cyclin E protein and its associated kinase were cell cycle dependent and maximal in late G_1 or early S phase (13, 42). We used immunofluorescence to examine more precisely the timing of cyclin E expression and its subcellular localization relative to both DNA synthesis and cyclin A expression. Additionally, we compared cell cycle-dependent changes in the subcellular localization of cyclin E with its overall level of expression and associated kinase activity. Serum-starved normal human fibroblasts were stimulated to reenter the cell cycle by refeeding with complete medium containing serum. No cyclin E nuclear staining was evident in serum-starved fibroblasts. Cyclin E was first detected in the nucleus 8 h after serum stimulation, and the fraction of cells expressing nuclear cyclin E peaked at 16 h (Fig. 4). This pattern of nuclear staining directly paralleled the expression of cyclin E protein as measured by immunoblotting (Fig. 5A) and the pattern of cyclin E-associated kinase activity (Fig. 5D). Thus, the cell-to-cell heterogeneity in cyclin E immunofluorescence in asynchronously proliferating cells is probably due to cell cycle-regulated expression of the cyclin E protein. In fact, we noted in an asynchronously proliferating cell population that those cells which could be morphologically identified as early-G_1 cells showed no cyclin E staining in the nucleus (Fig. 3C and D). Thus, cell cycle-regulated expression of cyclin E in the nucleus was not restricted to just the first cell cycle after release from serum starvation.

In these experiments the timing of cellular S phase was determined by measuring incorporation of tritiated thymidine into DNA. In Fig. 4B, the fractions of cells expressing cyclin E and cyclin A in the nucleus are compared with the levels of DNA replication at various time points. This showed that cyclin E nuclear staining preceded the onset of DNA synthesis and that the peak of cyclin E staining coincided with the G_1/S transition. This temporal relationship between cyclin E expression and DNA synthesis was confirmed at the single-cell level by incubating synchronized cells in BrdU and monitoring DNA synthesis by immunofluorescence detection of BrdU incorporation into nuclear DNA. By this assay as well, cyclin E nuclear staining preceded DNA synthesis (not shown). Moreover, in contrast to the pattern of cyclin D1 nuclear localization (2, 47), cyclin E nuclear staining did not decline as S phase began but remained elevated during early S phase. Indeed, most cells that could be costained with anti-BrdU and anti-cyclin E antibodies showed a spotted pattern of BrdU incorporation, which is typical of cells just entering S phase. Cells with a uniform pattern of BrdU staining, indicative of mid- to late S phase, showed lower levels of cyclin E in the nucleus. In contrast, cyclin A protein expression, associated kinase activity, and nuclear staining were first detected at the start of S phase and then continued to increase, peaking later in the cell cycle at about the G_2/M transition (Fig. 4B and 5A and D).

Normal human fibroblasts infected with the cyclin E-L cDNA-containing retrovirus expressed 10- to 20-fold more cyclin E than did control cells (Fig. 5B). The exogenously expressed cyclin E-L was present at approximately the same high level in serum-starved quiescent fibroblasts as in cells at the G_1/S transition. As a consequence of enforced expression, cyclin E was constitutively nuclear both in quiescent cells and throughout the cell cycle, showing that nuclear uptake of cyclin E is not cell cycle regulated (Fig. 3E and F). The subcellular localization of cyclin D1, on the other hand, seems to be cell cycle controlled. In contrast to the behavior of cyclin E, cyclin D1 disappears from the nucleus during DNA synthesis in mouse fibroblasts engineered to stably and continuously overexpress cyclin D1 protein (76). Despite the high levels of cyclin E protein in quiescent cells, cyclin E-associated kinase activity was not detected until 8 h after serum stimulation. This is about 4 h earlier than endogenous cyclin E-associated kinase activity (Fig. 5D) and is consistent with the extent of G_1 acceleration observed in these cells (61).

The above-described experiments showed that activation of cyclin E-associated kinase activity is negatively regulated in growth factor-deprived cells. However, we have been unable to explain this control of cyclin E activity in terms of pathways known to negatively regulate cyclin-Cdk complexes. First, the absence of cyclin E-associated kinase in quiescent cells was not due to an absence of cyclin E-Cdk2 complexes. Quiescent fibroblasts overexpressing cyclin E contained Cdk2 protein, albeit at a slightly lower level than that in proliferating cells (Fig. 6A). Moreover, cyclin E immunoprecipitates from these quiescent cells were immunoblotted with anti-Cdk2 antiserum

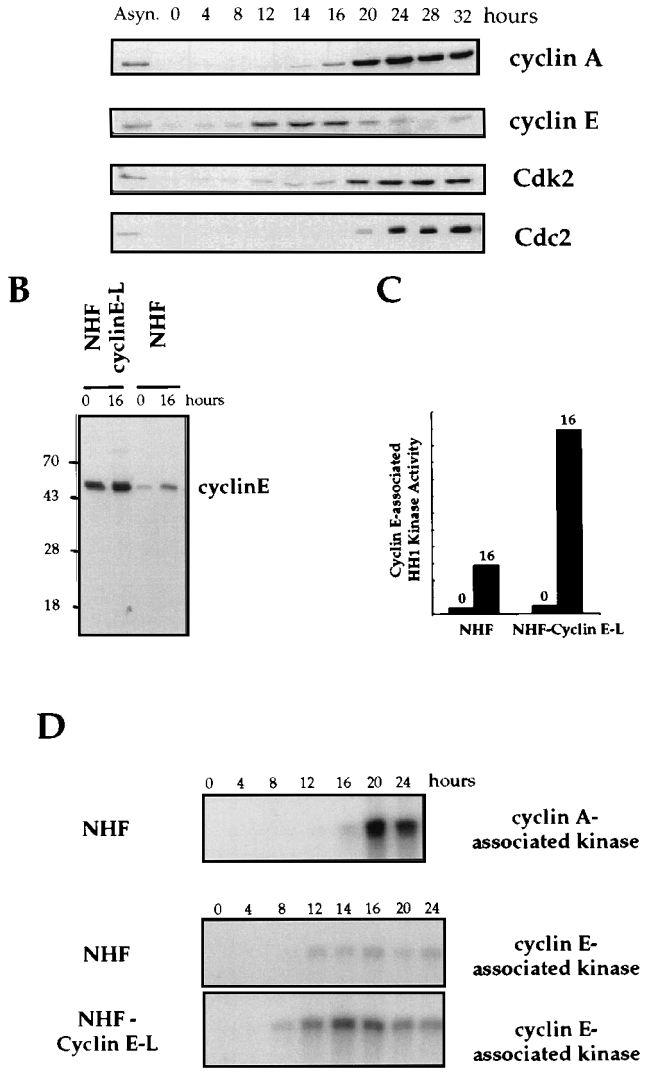

FIG. 5. Induction of cyclin E and cyclin E-associated kinase after serum stimulation of G_0-arrested normal human fibroblasts. (A) Immunoblot analysis. NHF cells were synchronized in G_0 by serum starvation for 72 h and then stimulated by adding fresh DMEM with 20% FCS. Total-cell extracts (from 2×10^5 cells) were prepared from cells at different times after serum stimulation, analyzed by SDS-PAGE, immunoblotted with specific antibodies against Cdks and cyclins. Asyn., extract from asynchronous culture. Long exposure of the Cdk2 blot, similarly to the blot in Fig. 6A, clearly shows the presence of this kinase in G_0 cells. (B) Immunoblot analysis of G_0-arrested and serum-stimulated NHF cells and NHF cells expressing cyclin E-L. G_0-arrested NHF cells expressing cyclin E-L were stimulated by adding fresh DMEM with 20% FCS. Total-cell extracts from 2×10^5 cells were prepared at 0 and 16 h after serum stimulation, and expression of cyclin E was compared with that of NHF cells. (C) Cyclin E-associated kinase in serum-stimulated NHF cells and NHF cells expressing cyclin E-L. Lysates (250 μl) from 10^6 cells either at quiescence (0 h) or at the G_1/S transition (16 h) were incubated with rabbit antiserum specific to cyclin E for 60 min on ice. Immunoprecipitates were collected on protein A-Sepharose, washed, and tested for histone H1 kinase activity (42). Phosphorylation of histone H1 (HH1) was quantitated. (D) Cyclin E and cyclin A-associated kinase activities. Extracts were prepared at the indicated times from quiescent and serum-stimulated NHF cells and NHF cells expressing cyclin E. Extracts were immunoprecipitated with cyclin A or cyclin E antiserum and tested for HH1 kinase activity.

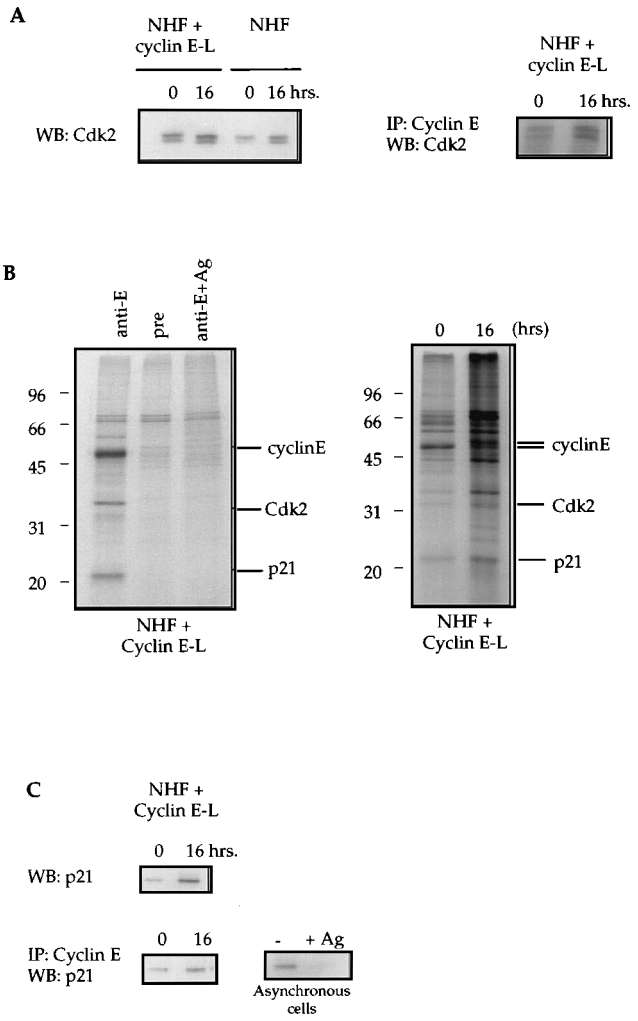

FIG. 6. Cyclin E-Cdk2 complexes in quiescent and proliferating normal human fibroblasts. (A) Expression of Cdk2 and association of cyclin E with Cdk2. Extracts were prepared from quiescent NHF cells expressing cyclin E-L and NHF cells expressing cyclin E-L at the G_1/S boundary (16 h post-serum stimulation). Shown are immunoblots (WB) using anti-Cdk2 antiserum of either whole-cell extracts or cyclin E immunoprecipitates. Cyclin E immunoprecipitates (IP) were prepared by incubation of cell lysates with cyclin E antibody that had been coupled to GammaBind Plus Sepharose (Pharmacia) by dimethylpimelimidate. (B) Composition of cyclin E-L complexes in quiescent (entire left panel and right panel, 0 h) and proliferating NHF cells expressing cyclin E-L (right panel, 16 h). Quiescent cells and cells stimulated with serum for 16 h were labeled with [^{35}S]methionine in the last 4 h (right panel, 16 h). Immunoprecipitates with anti-cyclin E antibodies (anti-E) were analyzed by SDS-PAGE. Control immunoprecipitations using preimmune serum (pre) and cyclin E antiserum plus cyclin E antigen (anti-E+Ag) are also shown. (C) Association of p21 with cyclin E. As in panel A, except that monoclonal anti-p21 antibodies were used in the immunoblots.

and found to contain no less than half as much Cdk2 as found in cyclin E immunoprecipitates from proliferating cells (Fig. 6A). A similar twofold change in the amount of cyclin E-associated Cdk2 was found by comparing cyclin E immunoprecipitates from extracts of quiescent and proliferating cells that had been labeled with [^{35}S]methionine (Fig. 6B). The small change in the abundance of cyclin E-Cdk2 complexes is unlikely to account for the complete absence of cyclin E-Cdk2 activity in serum-starved cells (Fig. 5C and D).

Second, as noted above, cyclin E is nuclear even when overexpressed in quiescent cells. Thus, cyclin E does not undergo

cell cycle-dependent transport into the nucleus, and this cannot explain the inactivity of cyclin E-Cdk2 complexes in quiescent cells. Third, the inactivity of these complexes was probably not due to the absence of activating phosphorylation on Cdk2 T-160. Phosphorylation of Cdk2 on T-160 causes a diagnostic mobility shift of the Cdk2 protein on SDS-polyacrylamide gel electrophoresis (PAGE) (26). Even in the quiescent state, cells overexpressing cyclin E had an increased amount of T-160-phosphorylated Cdk2 in comparison to that in control cells, and this activated form of Cdk2 was associated with cyclin E (Fig. 6A). Additionally, immunoprecipitates of cyclin E-Cdk2 complexes were not activated by incubation with purified Cdk2-activating kinase (not shown), the enzyme that phosphorylates Cdk2 on T-160 (21, 71, 86).

These observations suggested that Cdk2 is inactivated either by inhibitory tyrosine phosphorylation or by the inhibitory binding proteins p21 (27, 31, 102) and/or p27 (70, 95). Western blots (immunoblots) of cyclin E immunoprecipitates with antiphosphotyrosine antibodies did not show detectable differences in the amount of phosphotyrosine in cyclin E-associated Cdk2 in quiescent versus proliferating cells (not shown). Moreover, cyclin E-Cdk2 immunoprecipitates from quiescent cells were not activated by incubation with the *Drosophila* Cdc25 tyrosine phosphatase, although Cdc25 did modestly increase the activity of cyclin E-Cdk2 complexes harvested from proliferating cells (not shown). We detected about half as much p21 associated with cyclin E in extracts from quiescent cells compared with that in those from proliferating cells (Fig. 6C), consistent with the approximately twofold decrease in the total abundance of cyclin E-Cdk2 complexes in quiescent cells. Therefore, p21 did not appear to be the cause of cyclin E inactivity in quiescent cells. Furthermore, we were unable to detect by Western blot significant amounts of p27 in cyclin E immunoprecipitates under either condition (not shown). Finally, immunoprecipitation of cyclin E from [^{35}S]methionine-labeled cell extracts did not reveal any proteins that were specifically associated with cyclin E in quiescent cells (Fig. 6B) and therefore might account for the inactivity of this complex. The major cyclin E-associated proteins were Cdk2, p21, and a protein of 36 kDa, which probably corresponds to proliferating-cell nuclear antigen (PCNA) (104, 106). Additional bands are evident specifically in cyclin E immune complexes from proliferating cells, but these have not been identified. Whether these additional associated proteins contribute to the activity of the cyclin E-Cdk2 complex in proliferating cells is not known.

Antibodies to cyclin E injected in G_1 inhibit entry into S phase. The timing of cyclin E expression and activity and its physiological properties when overexpressed are consistent with it being an important regulator of the cell cycle during G_1. However, it has not been determined whether cyclin E is necessary for G_1 progression and entry into S phase in mammalian cells. To test this, we asked whether antibody-mediated knockout of cyclin E function interfered with entry into S phase. Normal human fibroblasts, IMR-90 cells, were arrested in G_0/G_1 by serum deprivation for 2 to 3 days. Four hours after reactivation with serum, the cells were microinjected with affinity-purified anti-cyclin E antibody or control rabbit immunoglobulins. In each experiment approximately 80 to 130 cells were microinjected within 10 min by using a computer-automated microinjection system (1, 66). Immediately after microinjection BrdU was added, and 24 h later cells were fixed and double stained for the injected antibodies and incorporation of BrdU. The pattern of nuclear staining for BrdU incorporation varied according to the position of the cell in S phase (24). Cell nuclei with a uniform and bright staining and those containing more than 20 bright spots (cell just entering S phase) were scored as BrdU positive. Microinjection of G_1-phase cells with anti-cyclin E antibody, but not with control rabbit immunoglobulins, inhibited DNA synthesis. The result of one experiment is shown in Fig. 7. DNA synthesis inhibition was due to the specific antibodies and not to a toxic component present in the antibody preparations, because preincubation of the cyclin E antibodies with recombinant cyclin E protein before microinjection reduced the inhibition of BrdU incorporation from 90 to 15% (data not shown). This was also supported by the fact that no inhibition of DNA synthesis was observed if the antibodies were injected after S phase had begun (see below). Finally, even if stored at −80°C, the antibodies slowly lost their ability to detect cyclin E in immunofluorescence experiments, and cell cycle inhibition after microinjection declined in parallel (not shown). Our data clearly indicate that inhibition of cyclin E function in G_1 inhibits the occurrence of S phase in human cells.

DNA replication is not inhibited by antibodies to cyclin E when injected during S phase. To pinpoint the time at which the cyclin E function is required for cell cycle progression, IMR-90 cells were injected with anti-cyclin E or control immunoglobulins in G_0 or at distinct times after serum readdition. At each time point cells were microinjected, and then BrdU was added. All samples were fixed 28 h after reactivation and then immunostained. In the noninjected cells, S-phase progression was also monitored by BrdU incorporation (Fig. 8A). Cells started to synthesize DNA between 12 and 16 h after serum addition, and by 24 h about 80% of the cells had incorporated BrdU. Panels B and C in Fig. 8 show that injection of anti-cyclin E antibody up to 8 h after reactivation resulted in a strong inhibition of DNA synthesis; at later times inhibition of DNA replication dramatically decreased. These results suggest that cyclin E plays an important role prior to but not during S phase.

We also compared the inhibitory effects of anti-cyclin E and D1 antibody microinjections at different times during G_1 (Fig. 8B). It has previously been shown that antibodies to cyclin D1 prevent DNA synthesis when injected at any time during G_1 until just before the start of DNA synthesis (2, 72). We reproduced similar results with both anti-cyclin E and anti-cyclin D1 antibodies. The only appreciable difference was when antibodies were injected into quiescent cells. Microinjections of anti-cyclin E antibody in G_0 cells were not very effective at blocking the subsequent S phase, whereas microinjections of anti-cyclin D1 antibody were strongly inhibitory. This difference was reproducibly observed in several experiments and could suggest that cyclin D1 acts at an earlier point in the cell cycle than cyclin E. Nevertheless, we have not ruled out the possibility the anti-cyclin E antibody is metabolized by the cell faster than the anti-cyclin D1 antibody and cannot efficiently inhibit DNA synthesis when injected too long before S phase starts.

Cyclin E, but not cyclin D1, is essential for the G_1/S transition in cells lacking pRb function. In human tumor cells lacking pRb function, cyclin D1 is expressed at low levels (5, 35, 46, 47, 55, 77, 87, 92), is dissociated from Cdk4 (4, 92), and is dispensable for progression through the G_1 phase of the cell cycle (46, 92). Indeed, microinjection of antibodies or antisense to cyclin D1 does not block the entry into S phase as it does in cells with wild-type pRb. We sought to investigate the effect of anti-cyclin E antibody in cells lacking pRb function. For this propose, we used fibroblasts expressing the SV40 large T antigen (IMR-90+LT cells) (92), in which pRb is inactivated by the viral protein, and the breast carcinoma cell line MDA-MB-468, which has a homozygous deletion of the pRb gene (3, 34, 43, 93). As a counterpart, we used normal IMR-90 fibro-

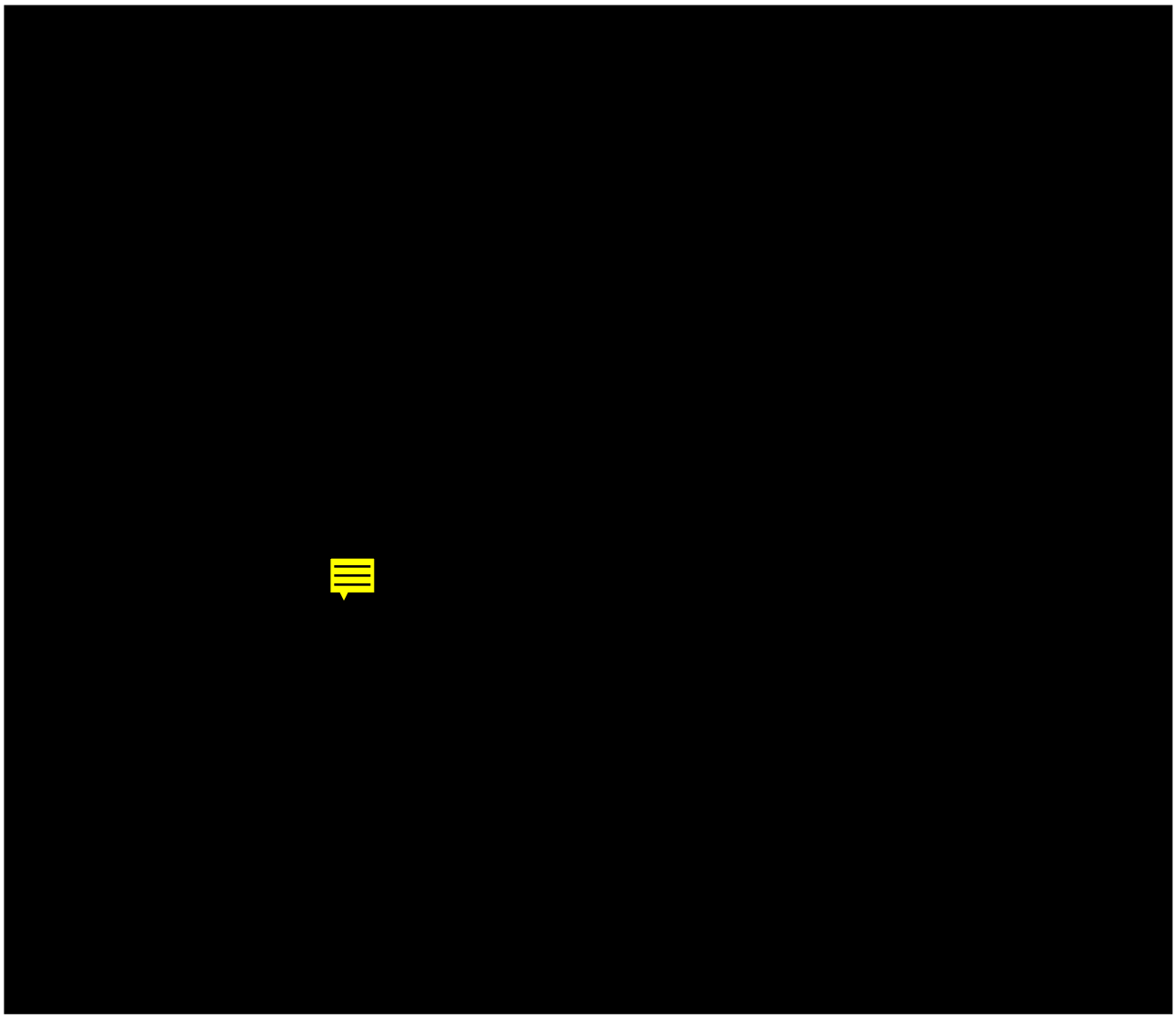

FIG. 7. Microinjection of anti-cyclin E antibodies in G_1 human fibroblasts inhibits DNA synthesis. Two representative fields of cells injected with affinity-purified anti-cyclin E antibody (A, B, and C) or with control purified rabbit antibodies (D, E, and F). Four hours after serum stimulation, microinjections were performed and BrdU was added. The cells were then incubated for a further 24 h, fixed, and processed for immunofluorescence. The two fields are stained with Hoechst 33258 (A and D), Texas red-conjugated anti-rabbit antibodies (B and E) to detect the cells injected with antibodies, and anti-BrdU antibody (C and F) to detect the cells replicating their DNA.

blasts and MCF-7, a breast carcinoma cell line having wild-type pRb (3, 43, 93). As already shown, G_1 cells lacking pRb function could readily enter S phase despite injection of affinity-purified antibody to cyclin D1. In contrast, cells injected with anti-cyclin E antibody arrested in G_1 regardless of whether pRb function is present (Fig. 9). IMR-90+LT cells were inhibited less efficiently than parental cells. This is probably due to the fact that large T antigen interferes with other cellular proteins besides pRb (e.g., p53, p107, and p130) and shows a pleiotropic effect on cellular growth (see reference 53 for a review).

DISCUSSION

Cyclin E, a G_1 cyclin. Cyclin E displays specific biochemical, molecular, and physiological properties which together establish that it has an essential and rate-limiting function in allowing cells to enter the S phase of the cell cycle. Cyclin E is a nuclear protein that attains its maximal level of expression at the G_1-to-S phase transition both in mitotically proliferating cells (13, 42) and in cells stimulated to reenter the cell cycle from a quiescent state (references 11 and 92 and this paper). Cyclin E binds to and activates the Cdk2 protein kinase, and in exponentially proliferating cells the assembly of catalytically active cyclin E-Cdk2 complexes is directly related to the abundance of the cyclin E protein (13, 42). Two forms of the cyclin E protein are expressed in most cells, and these forms are encoded by alternatively spliced mRNAs. The larger cyclin E protein (cyclin E-L) appears to be the predominant form in the cell types that we have analyzed, and it contains 15 amino acids at its amino terminus that are not present in the smaller form of cyclin E (cyclin E-S). Both forms of cyclin E are nuclear proteins, and both are expressed in similar patterns during the cell cycle.

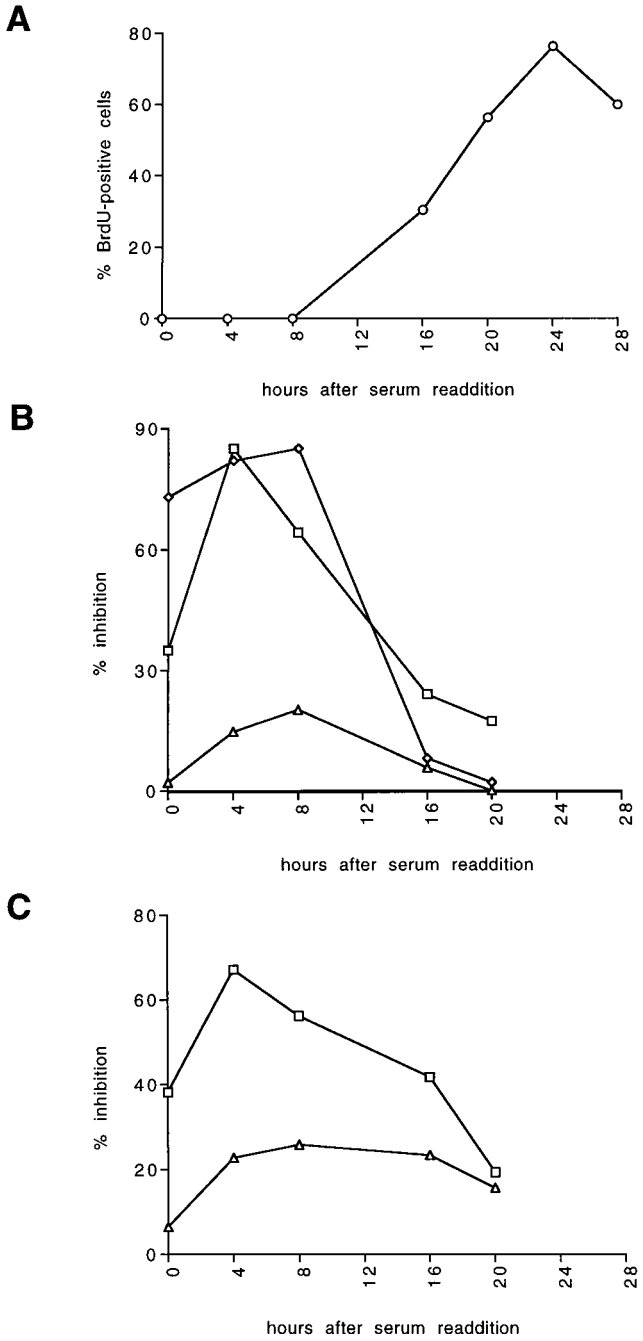

FIG. 8. Dependence of the DNA synthesis inhibition on the time point of injection of antibodies. (A) Human fibroblasts were arrested in G_0/G1 by serum deprivation. After reactivation the cells were incubated for 4 h with BrdU at the indicated times and then fixed and stained with anti-BrdU antibodies. Each value represents at least 200 counted cells per experiment. (B and C) Cells were microinjected at the indicated times with affinity-purified (AP) antibody to cyclin E (□) or to cyclin D1 (◇) or control rabbit immunoglobin G (△). BrdU was applied just after microinjection. At 28 h after serum stimulation, all the samples were fixed and immunostaining was performed as described in Materials and Methods. Panels B and C show the results of two independent experiments. BrdU-positive cells were scored as detailed in Results. The percent inhibition of BrdU incorporation was calculated as $[(N - I)/N] \times 100$, where N is the percentage of BrdU incorporation in noninjected cells and I is the percentage of BrdU in cells microinjected with antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU-positive cells that had not been injected. In each experiment about 100 injected cells (and a corresponding number of noninjected cells) were counted.

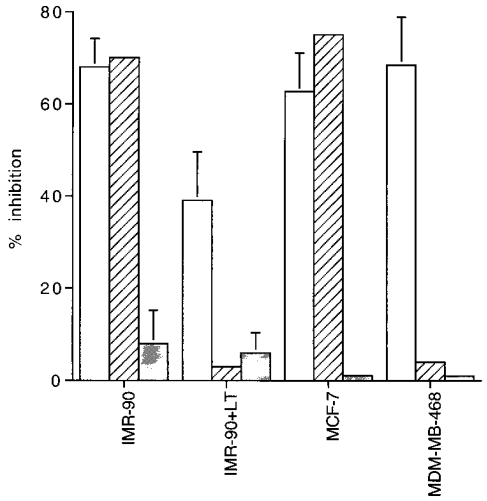

FIG. 9. Cyclin E, but not cyclin D1, is indispensable for the G_1/S transition in cells lacking pRb function. For each experiment 80 to 130 cells synchronized in G_1, as described in Materials and Methods, were injected with the indicated antibodies: affinity-purified anti-cyclin E (□) or D1 (▨) or rabbit immunoglobulin G (■). Approximately 24 h after microinjection, cells were fixed and processed for immunofluorescence. The percentage of DNA synthesis inhibition (± the standard error) from three independent experiments was calculated as described in the legend to Fig. 8.

Mammalian fibroblasts engineered to overexpress either cyclin E-L or cyclin E-S spend a smaller portion of the cell cycle in G_1 and a correspondingly longer portion in S and G_2/M. The extent of G_1 shortening appears to be determined by the time of cyclin E-Cdk2 activation during G_1. Thus, cells overexpressing cyclin E activate the cyclin E-Cdk2 complex 3 to 4 h earlier than control cells and shorten G_1 by the same amount. One possibility is that there is ordinarily a fixed length of time between activation of cyclin E and the start of S phase, a property consistent with the characteristics of the post-restriction point interval of G_1 (reviewed in reference 105). However, in cell lines overexpressing cyclin E we also observed that the extent of G_1 acceleration appears to be related to the level of cyclin E overexpression. Together these observations suggest that commitment to cell cycle progression (passage through the restriction point) may result in activation of the cyclin E-Cdk2 complex. Once cyclin E-Cdk2 is activated, the length of time until the onset of S phase may, to some degree, be determined by the level of cyclin E-Cdk2 kinase.

We have established here that cyclin E is also essential for the G_1-to-S phase transition in mammalian fibroblasts. The present study demonstrates that mammalian cells microinjected during G_1 with anti-cyclin E antibodies fail to enter S phase. In contrast, anti-cyclin E antibodies have no effect on cell cycle progression when injected into S-phase cells. Apparently, therefore, cyclin E function is required only prior to the onset of S phase. The mechanism by which cyclin E antibodies block cyclin E function in G_1 cells is not known, but they possibly prevent assembly or activation of the cyclin E-Cdk2 complex or the interaction of the holoenzyme with its substrates. These results are entirely consistent with those previously obtained in *Drosophila* embryos (40). Zygotic transcription of the cyclin E gene begins at cycle 17 of *Drosophila* embryogenesis. Cells transcribing cyclin E continue to proliferate, while those that do not express cyclin E arrest in G_0/G_1. The correlation between cyclin E expression and cell proliferation reflects an essential role for cyclin E in S-phase entry,

because all cells undergo G_1 arrest in cycle 17 in embryos homozygous for a cyclin E mutation. Finally, our results are in agreement with those obtained with *Xenopus* extracts (34a, 88) showing that p21 inhibition of DNA replication is rescued by addition of cyclin E (or cyclin A but not cyclin B or PCNA).

Regulation of cyclin E activity. As a consequence of its rate-limiting and essential role in G_1 progression, cyclin E activity is a common downstream target of antimitogenic signals that block the cell cycle in G_0/G_1. Cyclin E-Cdk2 is inactivated in G_1 by an inhibitory binding protein, p27, in epithelial cells exposed to transforming growth factor β (70, 84), in T cells treated with rapamycin (58), and in macrophages arrested in G_1 by cyclic AMP (37). DNA damage-induced G_1 arrest is also associated with inactivation of cyclin E-Cdk2 by a related inhibitory binding protein, p21 (8, 12). There is also evidence that phosphorylation of Cdk2 on threonine 14 and tyrosine 15 can inactivate this complex, but it is not yet known whether this is an important role in the physiological control of cyclin E-Cdk2 activity (11, 78).

Cyclin E-Cdk2 activity is also activated in response to mitogenic growth factors and cytokines. The first example of this was stimulation of human T-lymphocyte proliferation by the mitogen interleukin-2. Interleukin-2-starved lymphocytes arrest in G_1 with cyclin E-Cdk2 complexes inactivated by high levels of p27 (22, 58). Exposure of T lymphocytes to interleukin-2 causes elimination of p27 and activation of cyclin E-Cdk2. We have now shown that primary human diploid fibroblasts starved of serum growth factors also induce a pathway that prevents activation of cyclin E-Cdk2 complexes. Thus, mitogen-dependent activation of cyclin E-Cdk2 can be observed in both fibroblasts and lymphocytes and is likely to be a general feature of the process by which cells move from quiescence into an actively proliferating state. However, the biochemical mechanism by which cyclin E-Cdk2 is regulated by extracellular mitogens may not be the same in different cell types responding to different mitogenic signals. We have been unable to explain the inactivity of cyclin E-Cdk2 in quiescent diploid fibroblasts in terms of mechanisms previously observed in other experimental systems. Notably, we did not detect p27 in the inactive cyclin E-Cdk2 complexes. On the other hand, p21 is found in cyclin E-Cdk2 complexes in quiescent cells, but the amount of p21 was not noticeably greater than in active cyclin E-Cdk2 complexes harvested from proliferating cells. Nevertheless, we have not directly measured the stoichiometry of the p21-cyclin E complexes in quiescent and proliferating cells, and it remains possible that a change in this stoichiometry contributes to the regulation of cyclin E activity.

Metazoan G_1 cyclins. There is now clear evidence that three different cyclins, D1, E, and A, are all involved in G_1 progression or entry into the S phase of the cell cycle (reviewed in references 10, 32, and 82). There is also increasing evidence that each of these cyclins performs different functions to allow S phase to begin. Several studies have suggested that cyclin A is necessary at the end of G_1 to start DNA replication. D'Urso et al. (14) purified a factor from S-phase cells that was sufficient to allow replication of SV40 in extracts prepared from G_1 cells. This factor contained cyclin A, and it was then shown that addition of recombinant cyclin A to a G_1 cell extract was sufficient to allow SV40 DNA replication. It has been shown that cyclin A and Cdk2 colocalize with PCNA at subnuclear sites of cellular DNA replication (6, 23, 85) and that antibody-mediated inhibition of cyclin A or Cdk2 function, but not of cyclin B or Cdc2, inhibits DNA synthesis in human cells (25, 62, 63, 74, 96, 107).

Cyclin A and cyclin E appear to perform different functions in promoting the onset of S phase. This study shows that normal human fibroblasts, which have previously been shown to require cyclin A for S phase, also require cyclin E to enter S phase. In other words, in a single cell type both cyclins E and A are essential for the G_1-to-S phase transition. It has also been shown in newborn rat kidney cells that the actions of cyclin E and cyclin A are not redundant with respect to allowing cells to enter S phase. When newborn rat kidney cells are placed into suspension, they arrest in G_1 (28). These G_1-blocked cells contain active cyclin E-Cdk2 complexes, but they fail to express cyclin A. Moreover, enforced ectopic expression of cyclin A, but not cyclin E, allows these cells to enter S phase independently of cell anchorage (29). Thus, cyclin A is necessary for S-phase entry, and it cannot be replaced by cyclin E. We also show here that expression of cyclin E in the nucleus and the appearance of cyclin E-associated kinase activity occur earlier in the cell cycle than nuclear expression of cyclin A and accumulation of cyclin A-associated kinase activity. This appears to establish an order of action during the cell cycle, first cyclin E and then cyclin A, but it is not known whether this sequence is necessary for normal cell cycle progression. A possible candidate to link the consecutive activation of cyclin E and cyclin A is Cdc25A, a phosphatase required for entry into S phase in mammalian cells (33, 36). Cyclin E associates with, phosphorylates, and activates Cdc25A at the G_1/S transition (24a, 33). It is conceivable that activation of Cdc25 by cyclin E-Cdk2 is part of a positive-feedback loop, analogous to that described for the G_2/M transition. This loop could be important for the rapid activation of cyclin E-Cdk2 and perhaps also for the activation of cyclin A-Cdk2 by Cdc25A.

As in the case of cyclin E, microinjection of antibodies to another G_1 cyclin, cyclin D1, into human or murine fibroblasts inhibits entry into S phase (2, 72) and constitutive cyclin D1 overexpression accelerates the G_1 phase (72, 73). What is then the functional difference between cyclins E and D1? When directly compared in cells restimulated to reenter the cell cycle, both cyclin D1 mRNA and protein start to be expressed before cyclin E (11, 45, 57, 92). In addition, in contrast to cyclin E, cyclin D1 is present in the nucleus only during G_1 and never during S (2, 47) and enters and exits the nucleus about 4 h before cyclin E (94).

pRb and cyclin D1 pathways seem to be tightly connected and mutually regulatory (for a review, see references 18 and 83). For instance, in the absence of pRb function cyclin D1 protein is expressed at low levels (5, 35, 46, 47, 55, 77, 87, 92) and is dissociated from its catalytic partner Cdk4 (4, 92). In contrast, cyclin E protein levels (92) and cyclin E-Cdk2 complex (90) are not influenced by a lack of pRb. Furthermore, it has been shown that in human Rb^- cell lines (46, 92) and in mouse Rb null fibroblasts (45a), microinjection of antibodies or antisense plasmids to cyclin D1 does not prevent entry into S phase, in contrast to what happens in cells carrying a functional pRb. Similarly, overexpression of $p16^{Ink4A}$ or $p18^{Ink4C}$, two specific inhibitors of cyclin D-Cdk4 complexes (30, 80), prevents cell cycle progression only in pRb^+ cells (30, 16a, 45a, 51a, 79). Here we show that in Rb^- cells, cyclin E antibody microinjection prevents cell cycle progression, indicating that cyclin E is indispensable to the G_1/S transition regardless of whether pRb function is present.

In conclusion, we propose that cyclin E is a regulator of the G_1/S phase transition in human cells acting after cyclin D1 and before cyclin A. In contrast to cyclin D1, the requirement for cyclin E is independent of the pRb pathway.

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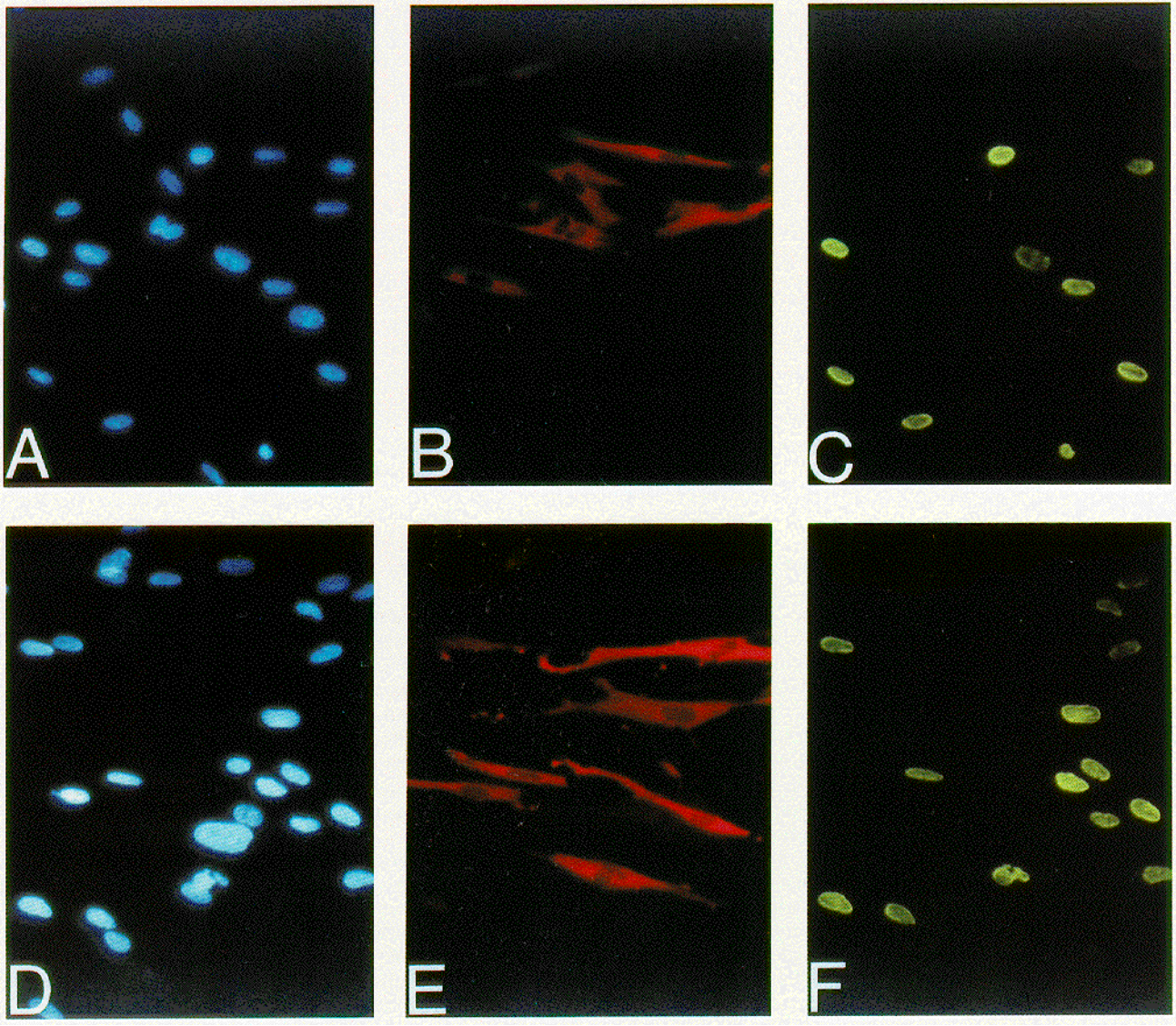
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