Overexpression of Cyclin E in the HC11 Mouse Mammary Epithelial Cell Line Is Associated with Growth Inhibition and Increased Expression of p27Kip1

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ABSTRACT

To elucidate the role of cyclin E in cell growth and tumorigenesis in mammary epithelial cells, we have used retrovirus-mediated transduction to generate derivatives of the nontransformed HC11 mouse mammary epithelial cell line that stably express a human cyclin E cDNA (HU4). These derivatives expressed two distinct forms of the exogenous cyclin E protein, which were about M, 50,000 and M, 42,000, thus corresponding to endogenous cyclin E proteins found in human cells. In contrast to results obtained previously in fibroblasts, overexpression of the HU4 cyclin E cDNA in HC11 cells was associated with an increase in cell size, lengthening of G1, and inhibition of both anchorage-dependent and -independent growth. Furthermore, when quiescent serum-starved cells were restimulated with serum, entry into the S-phase was delayed in the overexpressor cells. Under these conditions, there was also delayed induction in the expression of the endogenous cyclin E protein and in other events involved in the G1 transition. Despite the high level of expression of the exogenous cyclin E, the derivatives did not display increased cyclin E-associated in vitro kinase activity. The HC11 cells that overexpressed the exogenous cyclin E displayed an increase in the cyclin/cyclin-dependent kinase inhibitor p27Kip1 in both asynchronous exponentially dividing and synchronous cell populations. These findings indicate that increased expression of this cyclin E cDNA in HC11 cells inhibits rather than stimulates growth and that this may be due to increased expression of the inhibitor p27Kip1.

INTRODUCTION

Cyclins are a family of genes involved in the regulation of cell cycle progression in eukaryotes, through mechanisms that have been highly conserved during evolution (1–3). Originally described in marine invertebrates as proteins that displayed a remarkable periodicity in abundance during the cell cycle, they were subsequently identified in all eukaryotic cells from yeast to humans. They function essentially by controlling the timing of activation and the substrate specificity of a series of Cdks,4 which are sequentially activated during the cell cycle. Several cyclins and Cdks have been identified in mammalian cells. Specific cyclins bind to specific Cdks, thus activating their kinase activity. Each of these cyclin/Cdk complexes is activated at a specific point during the cell cycle and has a specific set of substrates (3–6).

G1 cyclins regulate the progression of cells through G1 and drive entry into S phase. Three D-type cyclins, D1, D2, and D3, act at mid-G1, by complexing with either Cdk4 or Cdk6 (1, 3). Cyclin E acts in late G1 by complexing with Cdk2 (7, 8). Several types of evidence indicate a rate-limiting role for these cyclins in G1 progression. Thus, overexpression of D-type or E cyclins accelerates the G1 to S transition and decreases cell size (9–13). On the other hand, antibodies to cyclin D1 (14, 15), cyclin D2 (16), or cyclin E (17) inhibit entry into S phase when injected into cells during the G1 phase of the cell cycle. However, their functions are not redundant (18). Thus, injection of antibodies, or treatment with antisense oligonucleotides to cyclin D1, blocks entry into S phase in cells with wild-type pRb but not in cells lacking pRb function (19, 20). In contrast, injection of anti-cyclin E antibodies arrests cells in G1, even in Rb– cells (17). Moreover, the cyclin D1/Cdk4 complex can phosphorylate in vitro the pRb-related protein p107 but not the pRb-related p130 (Rb2), whereas the contrary is true for the cyclin E/Cdk2 complex (21, 22).

Cyclin E is a nuclear protein originally isolated by screening human cDNA libraries for genes that could complement the loss of G1 cyclins in Saccharomyces cerevisiae (23, 24). As mentioned above, expression of cyclin E is cell cycle regulated and rises to a maximum level in late G1, after the increase in cyclins D1 and D2, and it associates with and activates Cdk2 (8, 25). The cyclin E/Cdk2 complex shows strong kinase activity shortly before cells enter S phase and leads to further phosphorylation of the pRb protein (26–30). The activity of the cyclin E/Cdk2 complex is further regulated by members of a family of CDIs, which include p21Waf1 (also designated Cip1, Pic1, Sdi1, and Cap20) and p27Kip1 (also called Ink and Pic2) (reviewed in Refs. 2 and 31–33).

Since cyclins play a pivotal role in controlling the order of events in the cell cycle, perturbations in their activity can have dramatic effects on cell cycle control and cell proliferation. Several lines of evidence indicate an involvement of cyclins and other cyclin-related genes in cancer. Most of this evidence relates to cyclin D1. This gene is frequently amplified and/or overexpressed in several types of human tumors (34–36). In contrast, the cyclin E gene is only rarely amplified in tumor cells (34, 37, 38). However, expression of the cyclin E protein is deregulated in several human cancers (39, 40). Thus, increased expression of multiple cyclin E-related proteins has been reported in human cancer cell lines and several types of primary tumors (37, 40). In breast cancer, these alterations in cyclin E expression have been associated with the stage and grade of the tumors (40).

To further address the role of cyclin E in growth control and tumorigenesis in mammmary epithelial cells, we have used retrovirus-mediated transduction to generate derivatives of the nontransformed HC11 mouse mammary epithelial cell line (41) that stably overexpress a human cyclin E cDNA (HU4) originally isolated from a glioblastoma cell line (23). It has been reported that stable overexpression of the same cDNA in rat and human fibroblasts induced a decrease in cell size, shortened G1, and reduced the requirement of serum for growth (10). The present study demonstrates that overexpression of the same cyclin E cDNA in HC11 cells inhibited, rather than stimulated, the growth of these cells. There was also an increase in the percentage of cells in G1 and a lengthening of the G1 to S transition. We show that these effects were associated with an increase in the level of expression of the CDI p27Kip1. The implications of these findings are discussed.
MATERIALS AND METHODS

Cell Culture. The HCC1 mouse mammary epithelial cell line was clonally derived from a spontaneously immortalized mammary epithelial cell culture originally established from a midterm-pregnant BALB/c mouse (41). HCC1 cells were grown and maintained in RPMI 1640 supplemented with 8% FBS, 10 ng/ml murine EGF, and 5 µg/ml bovine insulin (GM). For some experiments, cells were grown in the absence of these additional growth factors. The medium used to differentiate the cells contained RPMI 1640, 8% FBS, 1 µM dexamethasone, 5 µg/ml insulin, and 5 µg/ml bovine prolactin (DIP medium). HCC1 cells and derivatives were induced to produce β-casein by growing them, and maintaining them for 2 days at confluence, in GM. These “competent” cultures were then incubated for 2 days in the DIP medium. For cell synchronization studies, the cells were plated in GM at a density of 2 × 10^6 cells per 15-cm-diameter plate and cultured for 24–36 h. Then, after washing twice with RPMI 1640, the cells were incubated in RPMI, with no serum or growth factors, and cultured for an additional 72 h. The normal human breast and human breast cancer cell lines used in Fig. 8 were obtained from the American Type Culture Collection and cultured as recommended by the supplier. The human immortalized mammary epithelial cells 184B5 and 184A1 (a gift from M. Stapper, Lawrence Berkeley Laboratory, Berkeley, CA) were grown in supplemented MCDB 170 medium (Clonetech, San Diego, CA), as described previously (42). Rat-1 cells were obtained from J. M. Roberts (Fred Hutchinson Cancer Center, Seattle, WA) and cultured in DMEM medium plus 10% calf serum.

Construction of Retrovirus Vectors and Viral Transduction. The full-length human cyclin E cDNA (H4U), originally isolated from a human glioblastoma cell line (23), was subcloned into the HindIII site of the amphotropic vector PMV12 polylinker (PMV12pl; Ref. 43) in the sense orientation. To prepare infectious retrovirus particles, the resulting PMV12-cycE plasmid, or the control vector PMV12pl, were transfected into the Y2 ecotropic retrovirus packaging cell line (44) by the calcium phosphate precipitation method, as described previously (45). The transfected cells were selected by growth in 400 µg/ml of hygromycin (Boehringer Mannheim Corp., Indianapolis, IN) for 15 days. The hygromycin-resistant clones were then pooled and expanded. The viral supernatants, containing defective recombinant retrovirus particles carrying the respective sequences, were harvested, filtered, and then used to infect HC11 cells. The HC11 cells were infected with the retrovirus as follows. The cells were seeded at a density of 5 × 10^4 per 10-cm dish in GM. After 24 h, they were infected with 3 ml of medium containing serial dilutions of the viral stock solution and 8 µg/ml of Polybrene (Sigma Chemical Co., St. Louis, MO). After 4 h, 7 ml of fresh GM medium was added to each plate. Twenty four h later, the medium was replaced with fresh GM medium. After two days, the cells were trypsinized and replated into GM plus 600 µg/ml hygromycin (selection medium). Following selection, a resistant to hygromycin (hpa), several individual resistant clones were randomly isolated, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector. The cultures were then expanded and frozen as seed stocks. Resistant clones were grown in GM containing 300 µg/ml hygromycin. After 4–6 weeks in continuous culture, each clone was replaced from frozen seed stocks, since with serial passage they tended to lose expression of the exogenous cyclin E.

Growth Studies. The exponential doubling times and saturation densities were determined essentially as described previously (46). Cells were plated at a density of 1 × 10^4 cells per 35-mm-diameter well in triplicate. The number of cells per well was determined every 2 days for the subsequent 14 days, using a Coulter counter. Cells were refed with fresh medium every 2 days during this time. The doubling times were calculated from the initial exponential phase of the growth curves and the saturation densities from the plateau of the growth curves. Growth in soft-agar was also performed as described previously (46). For the bottom layer of agar, 1 ml of 0.5% agar in GM was placed in each 35-mm well of 6-well plates. Then 2 ml of 0.3% agar in GM containing 1 × 10^6 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days, and the final numbers of colonies larger than 0.1-mm diameter were determined.

Flow Cytometric Analysis. Cells were trypsinized, collected, and washed twice with PBS. Cell pellets were resuspended in 1 ml PBS and fixed in 5 ml of 70% ethanol and stored at 4°C. On the day of the analysis, cells were collected by centrifugation, and the pellets were resuspended in 0.2 mg/ml of propidium iodide in HBSS containing 0.6% NP40. RNase (1 mg/ml; Boehringer Mannheim) was added, and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 41 µm Spectra mesh filter (Spectrum, Houston, TX) and analyzed for DNA content on a Coulter EPICS 753 flow cytometer. The percentage of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program.

DNA Synthesis. Cells were plated in triplicate in 24-well plates at a density of 2 × 10^5 cells/well and incubated 24–36 h in GM medium. They were then rinsed twice with RPMI 1640 and grown in RPMI 1640 without serum or growth factors. After 72 h (time 0), complete GM was added to the cultures. At the indicated time points, the cultures were labeled for 1 h with [3H]thymidine (1 µCi/ml) (Amersham, Arlington Heights, IL) and then washed with ice-cold PBS and extracted with 10% cold trichloroacetic acid for 15 min on ice. After solubilization in 0.5 N NaOH, trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation counting.

Northern Blot Analysis. Cells from exponentially growing cultures were collected with a rubber policeman into 50-ml tubes, washed three times with ice-cold PBS, and then lysed by suspension in lysis buffer (3 M LiCl and 6 M urea) and homogenized for 1 min using a Polytron sonicator (Brinkmann Instruments, Westbury, NY). The tubes were kept on ice overnight at 4°C. After centrifugation, the pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 0.5% SDS and extracted with phenol/chloroform. The RNA samples (10 µg) were electrophoresed in 1% agarose-6% formaldehyde gels and blotted onto Hybond-N membranes (Amersham). The blotted membranes were preincubated in Church buffer (47) at 65°C and then hybridized with 3²P-labeled probes to human cyclin E, cyclin D1, or cyclin A for 16 h. The membranes were washed with 1X SSC (150 mM sodium chloride, 15 mM sodium citrate) containing 0.2% SDS for 20 min at room temperature, followed by 20 min at 65°C. After the final wash with 1X SSC at room temperature, the membranes were exsposed to Kodak XAR-5 film with intensifying screens at −70°C.

Immunoreagents. The polyclonal antibodies to cyclin D1, E, and A and to Cdk2 and Cdk4 were obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibodies to cyclin E (clone HEI2) and to pRb (clone G3-245) were purchased from PharMingen (San Diego, CA). The polyclonal antibodies to p21^Waf1 and p27^Kip1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Immune Complex Kinase Assay. Cdk enzyme assays were performed as described previously (48) with minor modifications. Cells were resuspended in kinase-lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin; all of these chemicals were from Sigma) and sonicated twice using a Sonifier Cell Disruptor (Ultrasonics, Inc., Plainview, NY). After centrifugation, clarified materials (50 µg) were incubated with protein A-Sepharose for 1 h at 4°C for preclaring in IP buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 20). Immunoprecipitations were carried out with 2 µg of the indicated antibody, and immunocomplexes were recovered with protein A-Sepharose for 1 h at 4°C. For H1 kinase activity, the protein A beads were washed four times with IP buffer, twice with washing buffer (50 mM HEPES (pH 7.5) and 1 mM DTT), and once with kinase buffer (50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate). The final pellet was resuspended in 50 µl of kinase buffer supplemented with 2 µg of Histone H1 (Boehringer Mannheim) and 5 µCi of [γ²³P]ATP (Amersham) and incubated for 15 min at 30°C. The reaction was stopped by the addition of 25 µl 2X-concentrated Laemmli sample buffer. The samples were separated on a SDS-10% polyacrylamide gel, and the phosphorylated histone H1 was visualized by autoradiography. For lysate mixing assays, lysates (50 µg) of cells collected at different time points after releasing from serum starvation were boiled for 5 min, and the precipitated proteins were cleared by centrifugation. The remaining supernatants were mixed with 50 µg of a cell lysate extracted from HCl1-V#1 cells at 20 h after releasing them from serum starvation. The mixed lysates were immunoprecipitated with polyclonal anti-cyclin E antibody and assayed for H1 kinase assay, as described above.

Immunoblotting and Immunoprecipitation. Protein extraction and immunoprecipitation were performed as described above for the H1 kinase assay.
Proteins from total cell lysates (50 μg) or immunoprecipitates (see above) were used for Western blot analysis, as described previously (46). Samples were electrophoresed by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Blots were then incubated with blocking buffer (50 mm Tris, 200 mm NaCl, 0.2% Triton X-100, and 3% BSA) for 90 min at room temperature. Different dilutions were used for different primary antibodies. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amersham).

To detect the exogenous human cyclin E in cyclin E/CDK2 complexes, 50 to 100 μg of cell lysates were immunoprecipitated with antiseraum to one subunit (i.e., anti-CDK2) and blotted with the monoclonal anti-cyclin E antibody. For the experiment shown in Fig. 2B, the polyclonal anti-cyclin E antibody was used for immunoprecipitation.

Immunohistochemistry. Cells cultured on glass slides were washed twice with PBS, fixed with cold methanol for 3 min at -20°C, and permeabilized with 0.5% NP40 in PBS for 5 min at room temperature. After blocking with goat or horse serum for 1 h at room temperature, cells were incubated with the polyclonal or the monoclonal anti-cyclin E antibody, respectively, overnight at 4°C. Different dilutions were used for different primary and secondary antibodies. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amersham). Secondary antibodies were detected by chemiluminescence or colorimetric methods.

RESULTS

Generation of Derivatives of the HC11 Mouse Mammary Epithelial Cell Line That Stably Overexpress Cyclin E. To address the role of cyclin E in cell cycle control and transformation in mammary cells, we overexpressed a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11. This cell line has been extensively used for studying the roles of different genes in mammary tumorigenesis (49, 50). A full-length human cyclin E cDNA (HU4), originally isolated from a human glioblastoma cell line (23), was subcloned into the HindIII site of the retroviral vector PMV12-polynkier (PMV12pl) in the sense orientation. The resulting PMV12-cycE plasmid or the control vector PMV12pl was transfected into the Y2 ectropic retrovirus packaging cell line (44) by the calcium phosphate precipitation technique, as described previously (45). The viral supernatants, containing defective recombinant Moloney murine leukemia viruses carrying the respective sequences, were harvested, filtered, and then used to infect HC11 cells. Following selection for resistance to hygromycin (hph), several resistant clones were isolated, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector (vector control cells; see “Materials and Methods” for details). In multiple transduction studies, the number of hph+ clones obtained with the PMV12-cycE construct was much fewer (only about 50%) than the number obtained with the control PMV12pl construct (data not shown). This finding suggested that overexpression of this cyclin E cDNA was toxic to many of the HC11 derivatives. Individual resistant clones were chosen for subsequent studies, rather than pools, since when pools of resistant clones were examined, we observed a progressive loss of cyclin E overexpression during serial passages (data not shown). This finding is consistent with the growth-inhibitory effects described below.

Expression of the exogenous cyclin E gene was verified by Northern blot hybridization and also by Western blot analysis using either a polyclonal anti-cyclin E antibody or a specific anti-human cyclin E monoclonal antibody. Fourteen of 18 clones examined showed high expression of the exogenous cyclin E and the corresponding mRNA. As shown in Fig. 1A, the level of the 5'-LTR-cyclin E-tkhp-LTR-3' transcript was very high in the cyclin E overexpressing clones compared to the level of the endogenous cyclin E mRNA in the vector control cells. The latter mRNA could be detected only after a longer exposure to the X-ray film.

The polyclonal anti-cyclin E antibody recognized at least two distinct forms of endogenous cyclin E in the exponentially growing parental and vector control HC11 cells, a major band at about M, 55,000 and a minor band at about M, 50,000. Using the same antibody in the cyclin E-overexpressing cells, we found a marked increase in the level of the M, 50,000 cyclin E band. In a previous study (10), introduction of the same cyclin E cDNA into fibroblasts also led to the expression of a M, 50,000 protein. With the polyclonal antibody, we also detected a faint M, 42,000 band in some of the overexpressor clones. However, when a specific antihuman cyclin E monoclonal antibody was used, a prominent M, 42,000 band was detected in all of the overexpressor clones, and the levels of this protein varied with the level of the M, 50,000 band (Fig. 1B). Otherwise indicated, in subsequent studies we used the polyclonal anti-cyclin E antibody since it enabled us to detect both the exogenous and the endogenous cyclin E proteins without losing any information, and the level of the M, 50,000 band also reflected the level of the M, 42,000 band.

To evaluate the subcellular localization of the overexpressed cyclin E protein, immunostaining for cyclin E was performed on both the control and cyclin E-overexpressing cell lines. The staining was almost exclusively nuclear in all of the cell lines examined, and the intensity was markedly increased in the cyclin E-overexpressing cells (Fig. 1C).

Effects of Cyclin E Overexpression on Cell Cycle Parameters and Cell Growth. To evaluate the phenotypic effects of cyclin E overexpression in HC11 cells, several parameters were examined, in parallel, in the parental cell line (HC11), two vector control cell lines (HC11-V71 and #2), and five cyclin E overexpressor clones (HC11-E#2, #8, #10, #12, and #13) that expressed various levels of the exogenous cyclin E (Fig. 1B).

The HC11-cyclin E clones did not display any morphological evidence of malignant transformation, and they did not form transformed foci. They were, however, morphologically different from the control cells since they were somewhat larger in diameter and showed cytoplasmic elongations, in both low- and high-density cultures, which were only rarely seen in the control cells (data not shown). When the cells were analyzed by forward angle light scatter, the results were consistent with an increase in cell size when compared to the control cells (Table 1).

The effects of increased expression of cyclin E on cell cycle distribution were analyzed by FACS analysis of exponentially growing cell cultures (Table 1). The cyclin E-overexpressing clones showed an increase in the percentage of cells in G1 and a reduction of cells in the S phase. Although these changes were not dramatic, they were reproducible and statistically significant (P < 0.01). The results shown in Table 1 were reproducible in more than 10 independent experiments.

We then compared the growth curves of monolayer cultures of the cyclin E-overexpressing clones and control cells. The overexpressing clones displayed a longer exponential doubling time than the control cells (about 18 h versus 15 h; Table 2). These differences, although not striking, were also reproduced in several experiments. Furthermore, there was about a 2-fold decrease in the saturation densities of the cyclin E-overexpressing clones when compared to the control cells (Table 2). Similar changes were also observed when cells were grown in medium containing only FBS (data not shown). Nor was there any evidence that the overexpression of cyclin E in HC11 cells decreased the requirement for growth factors, since, when either the control or cyclin E overexpressor cells were starved of serum, >90% of the cells arrested in the G0-G1 phase (data not shown).

The same cell lines were also assayed for anchorage-independent growth in soft agar. The cyclin E-overexpressing clones formed foci in soft agar with an efficiency about 2-fold higher than the corresponding control cells (Table 2). The foci formed in soft agar were stained with hematoxylin and eosin, and the number of foci was counted at 10 fields per agar dish. The results showed a significant increase in the number of foci in the cyclin E-overexpressing clones when compared to the control cells (Table 2). The results shown in Table 2 were reproducible in more than 10 independent experiments.
Constitutive overexpression of human cyclin E in the HC11 mammary epithelial cell line. A. Northern blot analysis of RNA from the parental HC11 cell line, three vector control cell lines (HC11-V#1, -V#2, and -V#6), and four cyclin E-overexpressing cell lines (HC11-E#2, -E#8, -E#10, and -E#12). Total RNA (10 μg) from the indicated cell lines was examined by Northern blot analysis. A 32P-labeled human cyclin E cDNA was used as the probe. Left, the position of the 5.0-kb (28S) and 2.0-kb (18S) RNAs. B. Western blot analysis of cyclin E protein. Whole-cell lysates were extracted from exponentially growing cultures of the indicated cell lines. Fifty μg of proteins were resolved by 10% SDS-PAGE and transferred to an Immobilon membrane. Duplicate blots were probed with polyclonal anti-cyclin E (top panel) and a monoclonal antihuman cyclin E (bottom panel) antibodies, and immunoreactive bands were detected by enhanced chemiluminescence. Left, molecular size markers in kilodaltons. Right, the positions of the endogenous (Endo) and the exogenous (Exo) cyclin E proteins. C. Nuclear localization of both endogenous and exogenous cyclin E proteins. Asynchronous exponentially growing HC11-V#1 vector control (a and b) and HC11-E#8 cyclin E overexpressor cells (c and d) were analyzed for cyclin E expression using the polyclonal (a and c) or the monoclonal (b and d) anti-cyclin E antibodies. The absence of reactivity in the vector control cells with the monoclonal antihuman cyclin E antibody (b) confirms the specificity of the staining. For additional details, see “Materials and Methods.”
Table 1  Flow cytometric analysis of cyclin E-overexpressing derivatives of the HCII mouse mammary cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G0-G1*</th>
<th>S</th>
<th>G2-M</th>
<th>Mean cell size</th>
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<td>HC11</td>
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<td>57.5</td>
<td>9.7</td>
<td>92.9</td>
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<tr>
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<tr>
<td>HC11-V#2</td>
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<td>56.3</td>
<td>20.0</td>
<td>92.1</td>
</tr>
<tr>
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<td>50.2</td>
<td>11.1</td>
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<tr>
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<td>26.5</td>
<td>17.0</td>
<td>93.5</td>
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*Exponentially growing cultures of the indicated cell lines were collected, and the DNA content was analyzed by flow cytometry.

Table 2  Growth properties of the cyclin E-overexpressing derivatives of the HCII mouse mammary cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>Saturation density × 10^6</th>
<th>Colony-forming efficiency (%)</th>
<th>Size &gt; 0.05 mm</th>
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<tr>
<td>HC11</td>
<td>16.4</td>
<td>8.7</td>
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<td>11.0</td>
<td>3.57</td>
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<td>0.05</td>
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*The doubling time corresponds to the initial exponential phase of cell growth (see "Materials and Methods" for details). The doubling time was significantly longer in the cyclin E overexpressor than in the vector control cells (P < 0.01).

**Effects of Cyclin E Overexpression on the G0-to-S Transition.** To further explore the effects of cyclin E overexpression on cell cycle parameters, we next used synchronized cell populations to analyze the interval from G0 to S phase in the cyclin E-overexpressing derivatives and control cells. The HC11-cyclin E clones and control clones were synchronized by serum starvation, which caused accumulation of more than 90% of both cell types in G0-G1, as assayed by FAC analysis (data not shown). FAC analysis of these cells at different points after refueling with complete growth medium showed that the parental cell line and the control clones entered the S phase at about 12 h after serum restimulation, whereas the clones that overexpressed cyclin E entered the S phase at about 15 h or later (data not shown). A similar difference was seen when the initiation of DNA synthesis was measured by [3H]thymidine incorporation (Fig. 3C). This difference was consistently observed for several cyclin E-overexpressing clones in control cells (data not shown). The G0-to-S progression was also slower for the cyclin E-overexpressing derivatives than the control cells when serum starved cells were stimulated to enter the cell cycle by refueling them with medium containing only EGF (data not shown).

Fig. 2. Effects of cyclin E overexpression on kinase activity. A, 50 µg of whole-cell lysates from exponentially growing cultures of the indicated cell lines were used to analyze the histone H1 kinase activity in anti-cyclin E immunoprecipitates. The immunoprecipitates were separated on 10% SDS-PAGE, transferred to an Immobilon membrane, and immunoblotted with the monoclonal anti-cyclin E antibody. Control whole-cell lysates (50 µg) from the HC11-V#1 (Lane 1) and the HC11-E#8 (Lane 4) cell lines are also shown. For additional details, see "Materials and Methods."
HC11 and vector control cells, the endogenous M, 55,000 protein was induced maximally at about 12 h, whereas in the cyclin E-overexpressing cell lines, maximal induction of this protein was delayed to about 16 h, and its total level of expression was also somewhat suppressed. As expected, in the latter clones, the expression of the lower molecular weight cyclin E proteins encoded by the exogenous cyclin E cDNA was constitutive throughout this time course (Fig. 3A). Maximum induction of the cyclin D1 protein was also delayed by about 4 h in the cyclin E overexpressor clones (Fig. 3B). Assays for cyclin E-associated kinase activity at different times after serum stimulation indicated that in the control cells this activity was maximal at about 12 h, whereas in the cyclin E overexpressor clones it was maximal at about 16 h, thus paralleling the delay in induction of the endogenous M, 55,000 cyclin E protein (Fig. 3A). A slight delay in the expression of cyclin A was also observed in the cyclin E overexpressor clones, but no significant differences were observed between the control and overexpressor clones in the expression of the Cdk2 and Cdk4 proteins (Fig. 3, A and B). However, the timing of Cdk2-associated kinase activity was delayed in the cyclin E-overexpressing cells (Fig. 3A), and a similar delay was observed also for the cyclin A-associated kinase activity (data not shown). In the control cell line, the pRb protein demonstrated a shift to the higher molecular weight hyperphosphorylated form (51) at about 8 h, whereas in the cyclin E overexpressor clones, this did not occur until about 12 h (Fig. 3B). This finding is consistent with the delayed induction of cyclin D1 and endogenous cyclin E and cyclin E-associated kinase activity (Fig. 3, A and B) in the overexpressor clones, since these events are implicated in the phosphorylation of the pRb protein (26, 27, 30). Thus, overexpression of exogenous cyclin E in the HC11 cells appears to have a feedback-inhibitory effect on several events involved in the G,-to-S transition, at least in cells synchronized by serum starvation. Furthermore, constitutive expression of the exogenous cyclin E cDNA did not increase cyclin E-associated kinase activity or enhance the G,-to-S transition.

The Cyclin E cDNA Used in This Study Reproduces the Effects Reported Previously When Overexpressed in Rodent Fibroblasts.

These observations raised the possibility that the inhibitory effects on cell growth, together with the lack of increased cyclin E-associated kinase activity, might be due to a defect in the cDNA used in these studies, or that the human cyclin E protein was unable to bind to and activate the murine Cdk2. However, the latter possibility seemed unlikely since human and mouse cyclin E show a high degree of homology (52) and human cyclin E has been reported to activate rodent Cdk2 (10, 12, 17, 18).

To verify the integrity of the cDNA used in this study, we prepared a pair of primers for the regions flanking the HindIII site of PMV12pl, into which the cyclin E cDNA had been subcloned, amplified this region by PCR, and sequenced the inserted cDNA. No difference was detected between the sequence of the cDNA present in our PMV12-cycE construct and the sequence originally reported for the HU4 cyclin E cDNA (Ref. 23; data not shown).

As mentioned above, the same human cyclin E cDNA was overexpressed previously in Rat1 fibroblasts and showed very different effects (10, 17). To further examine the integrity of the cyclin E cDNA used in this study, we obtained Rat1 fibroblasts and transduced them with the same retrovirus constructs used to transduce the HC11 cells. Several independent pools of Rat1 cells transduced with either the PMV12pl or the PMV12-cycE constructs were selected by growth in hygromycin and then analyzed. Western blot analysis, using the polyclonal anti-cyclin E antibody, showed increased expression of a Mr, 50,000 cyclin E protein in the latter derivatives, and this was associated with an increase in the amount of cyclin E-associated histone H1 kinase activity (Fig. 4). Using the monoclonal antibody specific for human cyclin E, we also detected the Mr, 42,000 band seen in derivatives of HC11 cells (data not shown). When exponentially growing cells were examined by FACS analysis, unlike the HC11-cyclin E derivatives (Table 2), the cyclin E-overexpressing derivatives of Rat1 cells showed a decrease in the proportion of cells in G, (36%...
overexpression of human cyclin E in rat fibroblasts. Exponentially growing Rat1 fibroblasts were infected with the same virus supernatants used to infect the HC11 mouse mammary cells. Top panel, expression of cyclin E was determined by Western blot analysis in whole-cell extracts (50 μg) from exponentially growing cultures of the Rat1 parental cell line, Rat1 vector control cell line, and Rat1/cyclin E-infected cells. The higher band corresponds to the endogenous M, 52,000–55,000 form of cyclin E, and the smaller band corresponds to the M, 50,000 form of the exogenous cyclin E, which is highly overexpressed in the Rat1/cyclin E cells. In the bottom panel, the histone H1 kinase activity in anti-cyclin E immunoprecipitates was assayed as described in Fig. 2.

versus 58%) and an increase of cells in S phase (48% versus 30%), thus reproducing the results reported previously (10). Thus, we excluded the possibility that the above-described effects in HC11 cells were due to a defect in the cyclin E cDNA used to transduce these cells. Furthermore, we have found that overexpression of the same cyclin E cDNA in NIH3T3 cells led to increased cyclin E-associated histone H1 kinase activity (data not shown). Therefore, human cyclin E is capable of activating the murine Cdk2.

Cyclin E Overexpression Is Associated with Increased Expression of p27Kip1 in Mammary Epithelial Cells. The slower G1-to-S transition in serum-starved and restimulated HC11 cyclin E overexpressor cells could be explained, in part, by the delayed induction of endogenous cyclins D1 and E and the associated delay in phosphorylation of the Rb protein (Fig. 3). However, it was not apparent why, despite the fact that these cells constitutively express high levels of the exogenous cyclin E, they did not display an increase in cyclin E-associated kinase activity (Figs. 2 and 3). As mentioned above, this was not due to the inability of human cyclin E to bind to or activate murine Cdk2. The endogenous M, 50,000 and M, 42,000 cyclin E proteins were readily detected by immunoblotting in cell extracts from either exponentially dividing or synchronized HC11 overexpressor cells, following immunoprecipitation with either anti-cyclin E (Fig. 2) or anti-Cdk2 (Fig. 5B) antibodies.

Several CDIs have been identified recently (31, 32). Amongst them, the CDIs p21Waf1 (also called Cip1, P1C1, Sdi1, and Cap20) and p27Kip1 (also called Ink4 and P1C2) preferentially bind to and inhibit the cyclin E-Cdk2 complex (53–56). Therefore, we examined the possibility that these proteins might play a role in inhibiting the activity of the exogenous cyclin E in the HC11 derivatives. Western blot analysis of exponentially dividing or synchronized cells indicated that the p21Waf1 protein was barely detectable in both the control and cyclin E overexpressor HC11 cells (data not shown). When serum-starved and restimulated cells were examined for the expression of the p27Kip1 protein, the vector control cells displayed a moderate level of this protein, which decreased at about 6 h after serum stimulation (Fig. 5A). The cyclin E overexpressor clones displayed a relatively high level of this protein at time 0, and this level rose to even higher levels at 4–6 h after serum stimulation and then declined somewhat. The results of a typical experiment are shown in Fig. 5A for the cell lines HC11-V#1 and HC11-E#8, but similar results were obtained with additional cyclin E overexpressor clones (HC11-E#2, HC11-E#12, and HC11-E#13; data not shown). Exponentially dividing cultures of the cyclin E-overexpressing clones also displayed increased expression of the p27Kip1 protein when compared to the control clones (Fig. 6). Furthermore, with serial passage, the cyclin E overexpressor clones tended to display decreased levels of expression of the exogenous cyclin E, and this was associated with a decline in the level of the p27Kip1 protein (data not shown).

We also obtained evidence that the p27Kip1 protein was complexed to the exogenous cyclin E (Fig. 5B). Following restimulation of serum-starved cells, extracts were obtained at 0, 12, and 20 h, and immunoprecipitates were prepared with an antibody to Cdk2 or an antibody to p27Kip1. These precipitates were then fractionated by SDS-PAGE and immunoblotted with the monoclonal antihuman cyclin E antibody. The results obtained (Fig. 5B) are consistent with the presence of a complex that contains the human cyclin E, murine Cdk2,
and p27Kip1 protein in the cyclin E overexpressor but not the control HC11 clones. Evidence for this cyclin E-p27Kip1 complex was also obtained in exponentially dividing HC11-E8#8 cells (Fig. 5C). We found that the amount of the exogenous cyclin E protein in the immunoprecipitates obtained with the p27 antibody, from extracts of the cyclin E overexpressor cells, was approximately equivalent to the total amount of exogenous cyclin E present in the corresponding whole-cell lysate (data not shown). Thus, it appears that most, if not all, of the exogenous cyclin E is complexed with p27Kip1. Therefore, the cyclin E overexpressor cells express much higher levels of p27Kip1, and this inhibitory protein is also associated with the exogenous cyclin E. We could not accurately assess the amount of the endogenous cyclin E protein in these immunoprecipitates since it was obscured by the IgG band.

The p27Kip1 protein is heat stable (54, 57). To verify the presence of increased amounts of a heat-stable inhibitory activity in the cyclin E-overexpressing HC11 cells, cell lysates were collected from HC11-V#1 control cells and HC11-E8#8 overexpressor cells at different intervals during the G1-to-S transition. The lysates were boiled for 5 min, and the precipitated proteins were removed by centrifugation. The remaining supernatant fractions were mixed with fixed amounts (50 μg) of cell lysate collected from control cells at a time point when cyclin E-associated kinase activity was very high (20 h). This mixture was then assayed for cyclin E-associated kinase activity (see "Materials and Methods"). Although not all of the p27Kip1 protein was recovered after boiling the cell extracts (data not shown), heat-stable inhibitory activity was present in cell lysates collected at time zero from both control cells and cyclin E-overexpressing derivatives (Fig. 7). This inhibitory activity progressively decreased in control cells but increased at time points up to 16 h in the cyclin E-overexpressing cells, thus resembling the pattern of expression of the p27Kip1 protein shown in Fig. 5A. Since p27Kip1 is not the only heat-stable CDI, it is possible that other CDIs also contribute to this inhibitory activity. However, Western blot analyses failed to detect the recently identified p57Kip2 CDI protein (58, 59) in the extract of the HC11 control or cyclin E overexpressor cells (data not shown).

Cyclin E and p27Kip1 Expression Levels in Human Breast Cell Lines. Increased expression of cyclin E proteins has been reported in several types of human tumors, including breast cancer (34, 39, 40), and several human breast cancer cell lines (37). Therefore, it was of interest to examine whether deranged expression of the endogenous cyclin E in human breast cell lines was associated with increased expression of p27Kip1. To test this idea, we examined, by immunoblotting, the levels of expression of cyclin E and p27Kip1 proteins in several human cell lines originally derived from normal human mammary epithelial cells or breast carcinomas. As shown in Fig. 8, only the major M, 52,000 form of cyclin E was expressed in the three nontumorigenic, immortalized mammary epithelial cell lines 184B5, 184A1 (42), and MCF-10F (60). The slight shift in this band is probably due to phosphorylation, as reported previously (25). On the other hand, all of the breast cancer cell lines examined (MCF-7, ZR-75-1, T47D, DU4475, and BT-549) showed increased expression of this M, 52,000, as well as the presence of one or more lower molecular weight cyclin E-related proteins, including the M, 50,000 and M, 42,000 forms seen in the cyclin E-overexpressing derivatives of the HC11 cell line (Fig. 8). These lower molecular weight, cyclin E-related proteins were also observed in the Hs578Bst and HBL-100 cell lines. The Hs578Bst cell line was derived from normal breast tissue peripheral to a carcinosarcoma, and because it is fibroblast like, it might be myoeppithelial in origin (61). Although HBL-100 is an epithelial cell line originally derived from normal human mammary epithelium, it contains a tandemly integrated SV40 virus genome and is tumorigenic in nude mice (Refs. 46 and 62; ATCC catalog). It is of interest that the level of the p27Kip1 protein was much higher in all of the breast cancer cell lines examined (Fig. 8, Lanes 8–12) than in the nontumorigenic, immortalized human mammary epithelial cell lines (Fig. 8, Lanes 3–5). Thus, aberrant expression of the endogenous cyclin E in these human cell lines is associated with increased expression of p27Kip1.
and Methods”) and the level of β-casein expression was determined by immunoblotting. The parental HC11 cells displayed a low level of spontaneous β-casein expression that was increased by the DIP induction (Fig. 9, compare Lanes 1 and 9). The cyclin E-overexpressing derivatives of HC11 cells showed a high level of spontaneous production of β-casein (Fig. 9), which was not further increased by hormone induction (data not shown). These findings suggest a possible relationship between cyclin E overexpression and differentiation in mouse mammary epithelial cells, although additional markers of differentiation remain to be examined.

DISCUSSION

To further address the role of cyclin E in mammary tumorigenesis, in the present study we developed derivatives of the normal mouse mammary epithelial cell line HC11, which stably overexpress a human cyclin E cDNA (HU4). We found that this causes the expression of two different forms of the cyclin E protein, a major band at about M, 52,000 and a minor band at about M, 42,000 (Fig. 1). The appearance of the smaller band was reported previously in derivatives of fibroblasts that overexpress the same human cyclin E cDNA, but this was seen only occasionally and was attributed to proteolytic degradation of the overexpressed M, 50,000 band or to translation initiation at an internal methionine residue (10). With the use of a specific antihuman cyclin E antibody, we were able to demonstrate that the M, 42,000 cyclin E protein was consistently expressed in parallel with the M, 50,000 band in the derivatives of the HC11 cells. Furthermore, in some of these derivatives, we could detect the M, 42,000 protein in clones that contained only low levels of the M, 50,000 protein (Fig. 1), suggesting that it is not simply a minor degradation product. A second human cyclin E cDNA has been cloned recently that codes for a M, 52,000 protein (17), and evidence has been obtained that the M, 52,000 and M, 50,000 proteins are encoded by alternatively spliced mRNAs (17). Overexpression of the M, 52,000 cyclin E form in fibroblasts has been reported to cause the same effects as overexpression of the M, 50,000 form (17). A M, 43,000 splice variant of human cyclin E was identified previously in several human cell lines (64).

We suggest that the M, 42,000 band observed in the HC11/cyclin E-overexpressing derivatives (Fig. 1) is due to further splicing of the overexpressed cyclin E mRNA, since this mRNA contains both potential splice-donor and splice-acceptor sites (64). The existence of multiple cyclin E-related proteins that range in size from M, 52,000 to about M, 35,000 has been described previously in human cancer cells (Refs. 37 and 40; Fig. 8). The precise origin of these multiple forms of the cyclin E protein and their functional significance is not, however, apparent at the present time.

The present study demonstrates that overexpression of the same cyclin E cDNA can exert distinct biological effects in different cell types. Thus, whereas previous studies (10, 17) found that stable overexpression of the HU4 cyclin E cDNA in rat or human fibroblasts shortened G1, decreased cell size, and enhanced growth, we found that stable overexpression of the same cDNA in the HC11 cells lengthened G1, increased cell size, and inhibited growth (Tables 1 and 2; Fig. 3). Our findings are reproducible since they were seen with several clonal derivatives of HC11 cells that overexpress this cyclin E cDNA and were not seen in vector control clones (Tables 1 and 2). Furthermore, sequencing studies indicated that the present results are not due to mutations in this cDNA that might have occurred during construction of our PMV12-cycE plasmid. In addition, when we introduced the same construct into rat fibroblasts, we reproduced results obtained previously by Ohtsubo et al. (10, 17).

Further studies are in progress to determine whether the effects seen with the HC11 cells occur with other mammary epithelial cells. The divergent effects of this cyclin E cDNA in different cell types are not surprising since there are several other examples in which cell context influences the action of other genes involved in growth control. Thus, instead of enhancing growth, in certain cell lines the ras (65, 66), fos (67, 68), and jun (69) oncogenes can inhibit growth and induce differentiation; and the bcl-2 (70) and protein kinase Cβ (71) can have reciprocal effects in different cell types. Moreover, we reported recently that stable overexpression of a cyclin D1 cDNA in the HBL-100 human mammary epithelial cell line inhibits growth (46), whereas overexpression of the same cDNA in rat fibroblasts enhances their growth (9).

We believe that the most likely explanation for why the HC11/cyclin E derivatives have a prolonged G1 phase and display growth inhibition is that stable overexpression in these cells of this human cyclin E cDNA induces, either directly or indirectly, the expression of the CDI protein p27Kip1. The increased expression of this inhibitory protein was seen in the HC11/cyclin E cells when they were in continuous exponential growth and also in cultures synchronized by serum starvation and refeeding (Figs. 5 and 6). It was also a reproducible finding in several cyclin E-overexpressing clones and was not seen in the vector control clones. This interpretation is consistent with the fact that although these derivatives displayed a marked increase in the levels of the exogenous cyclin E proteins, extracts of these cells did not display an increase in cyclin E-associated kinase activity when compared to extracts from control cells (Fig. 2). Extracts of the overexpressor cells also displayed an increase in inhibitory activity in in vitro assays for cyclin E-associated kinase activity (Fig. 7). In addition, both the exogenous cyclin E protein and the p27Kip1 protein
could be detected in cyclin E/Cdk2 immunoprecipitates obtained from the overexpressor cells (Fig. 5).

Although it was originally reported that p27Kip1 is expressed at a constant level in murine fibroblasts (55), subsequent studies have shown that the level of p27Kip1 increases in serum-starved cells and is down-regulated by growth factors (72, 73). Moreover, when quiescent T lymphocytes are stimulated to proliferate by the addition of interleukin 2, the level of this protein decreases (74, 75). This decrease in p27Kip1 is prevented by rapamycin, a potent immunosuppressant that inhibits the G1-to-S progression, presumably by preventing activation of the cyclin E/Cdk2 complex (75). Moreover, p27Kip1 accumulates in Mv1Lu mink epithelial cells arrested in G0/G1 due to cell-cell contact or treatment with transforming growth factor β (57, 76, 77), and overexpression of exogenous p27Kip1 in mink Mv1Lu or human Saos-2 osteosarcoma cells arrests cells in G1 (54, 55). There is also evidence that dislocation of p27Kip1 from the cyclin E/Cdk2 complex is essential for the activation of kinase activity and that this is also prevented by transforming growth factor β treatment (57, 76). All of these findings are consistent with our hypothesis that the increased levels of p27Kip1 in the HCl1/cyclin E-overexpressing cells may explain several aspects of their phenotype.

We further hypothesize that the increased expression of p27Kip1 in the HCl1/cyclin E-overexpressing cells is a manifestation of a positive feedback loop between cyclin E and p27Kip1, which is present in HCl1 cells but may be absent or have a different set point in fibroblasts. Consistent with this hypothesis is the fact that rat fibroblasts that stably overexpress the same human cyclin E cDNA do not display an increase in the p27Kip1 protein (data not shown). The increase in p27Kip1 in the HCl1-cyclin E overexpressor cells might protect these cells against potentially toxic effects of cyclin E overexpression, thus enhancing their viability although they grow more slowly. It is of interest that with prolonged serial passages, the HCl1-cyclin E10 and E112 clones displayed a loss of expression of the exogenous cyclin E, and this was associated with decreased expression of p27Kip1 (data not shown). Further studies are required to definitively establish that overexpression of cyclin E directly induces p27Kip1 expression in HCl1 cells and that this association is not simply due to cell selection.

We should also emphasize, however, that the unusual effects seen in the present study might be peculiar to ectopic expression of specific cyclin E proteins and may not play a role in normal cell physiology. Thus, the parental HCl1 cells mainly express a M1, 55,000 form of cyclin E and the M2, 50,000 form is expressed at a very low level (Fig. 1). Moreover, only the M5, 50,000 band undergoes specific cell cycle regulation, both in terms of its level of expression and phosphorylation (Fig. 3). On the other hand, the cyclin E-overexpressing derivatives express high levels of the M5, 50,000 and M6, 42,000 human cyclin E proteins. In addition, the ectopic cyclin E proteins are expressed constitutively throughout the cell cycle, in contrast to the endogenous cyclin E proteins (Fig. 3). This temporal difference might also perturb normal cell cycle regulatory events and the expression of p27Kip1.

The present model system may, however, be instructive for revealing regulatory loops that apply in certain circumstances. Thus, as discussed above, certain tumor cells and some normal cell lines often display dysregulation in the expression of their endogenous cyclin E gene, and these cells also accumulate lower molecular weight cyclin E proteins. Some of these proteins might be defective in binding to and activating Cdk2, like the M4, 42,000 protein (64), but they could also play a role in feedback regulation of cell cycle progression. It is of interest that the HCl1 derivatives that express the exogenous cyclin E cDNA display not only increased expression of the endogenous p27Kip1 but also decreased expression of their endogenous cyclin E protein (Figs. 1 and 3). Additional studies are required to determine if this represents an independent negative feedback loop in the control of cyclin E expression or is a secondary effect due to changes in cell cycle control.

Finally, the findings in the present study may be relevant to human breast cancer since we have found that a series of tumorigenic human breast cancer cell lines express a high level of p27Kip1 and that the same cell lines display increased expression of lower molecular weight forms of cyclin E (Fig. 8). The high level of expression of p27Kip1 is surprising since this protein is thought to act as a tumor suppressor (2). Thus, it is possible that our hypothesis of a positive feedback loop between cyclin E and p27Kip1 applies to these cell lines. Studies are in progress to further evaluate the significance of this association in human breast cancer.

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CYCLIN E OVEREXPRESSION IN MOUSE MAMMARY CELLS

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Overexpression of Cyclin E in the HC11 Mouse Mammary Epithelial Cell Line Is Associated with Growth Inhibition and Increased Expression of p27Kip1

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