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Induction of Mammary Gland Hyperplasia and Carcinomas in Transgenic Mice Expressing Human Cyclin E

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Deregulated expression of several cell cycle regulatory genes has been demonstrated to be associated with cancer. In particular, a strong correlation has been established between inappropriate cyclin E expression and human breast cancer. To determine the ability of cyclin E to play a causative role in mammary tumorigenesis, regulatory sequences from the ovine β-lactoglobulin gene were utilized to specifically target expression of human cyclin E to the mammary glands of pregnant and lactating mice. Lactating mammary glands of transgenic mice expressing cyclin E contained areas of hyperplasia, primarily papillary projections of hyperplastic cells, which were rarely observed in lactating glands of control mice. Over 10% of female cyclin E transgenic mice have developed mammary carcinomas, with latencies ranging from 8 to 13 months. Tumor analysis revealed the presence of transgene-specific cyclin E RNA and protein, as well as cyclin E- and cdk2-associated kinase activity, suggesting that cyclin E is likely a contributing component of tumorigenic progression in this model system.

The regulation of critical transitions through the cell cycle is mediated by distinct protein kinase complexes, each composed of a cyclin regulatory and a cyclin-dependent kinase (cdk) catalytic subunit (8, 35, 37, 41). In mammalian cells, several structural classes of cyclins (A to H) have been identified and have been demonstrated to reach maximal abundance during particular phases of the cell cycle (8, 35). Individual cyclins associate in various combinations with specific members of the cdk family, which in mammalian cells contains at least six cdks (28, 35). The temporal regulation of cellular events necessary for orderly cell cycle progression is thought to be achieved by the sequential activation of different cyclin-cdk complexes throughout the cell cycle.

Much evidence indicates that progression through the G_1 phase of the cell cycle is dependent upon the activity of G_1 cyclins, which include the D-type cyclins and cyclin E. The D-type cyclins reach maximal levels of expression and form functional kinase complexes with cdk4 or cdk6 during mid-G_1 phase (3, 25–27, 44, 50), whereas cyclin E is expressed and associates with cdk2 in an active complex near the G_1/S boundary (10, 19). Inhibition of cyclin D1 by microinjection of antisense plasmids or antibodies during mid-G_1 phase prevents entry into S phase (2, 36). Similarly, microinjection of cyclin E antibodies prior to S phase inhibits the occurrence of S phase (32), as does inhibition of cdk2 activity by dominant negative or antibody microinjection methods (34, 45, 46). Overexpression of cyclin D1 or E accelerates progression through G_1, reduces growth factor requirements, and diminishes cell size (31, 36, 39, 49), while an additive effect on G_1 phase shortening is observed when both cyclins D1 and E are overexpressed in the same cell (38). These data suggest that the D-type cyclins and cyclin E are essential for progression through G_1 phase, controlling events that are rate limiting for the G_1/S transition.

Since enforced perturbations in the expression or activity of G_1 cyclin-cdk complexes cause disruption of normal G_1/S phase progression, loss of normal growth control could potentially result from inappropriate G_1 cyclin function. This hypothesis is supported by several studies demonstrating an association between deregulated G_1 cyclin gene expression and oncogenesis. Overexpression of cyclin D1 as a result of chromosomal rearrangement or gene amplification has been observed in several human tumor types (13). Cyclin D1 has also been shown to cooperate with Ha-ras in the transformation of primary cells (12, 22), to cooperate with myc in the generation of B-cell lymphomas in transgenic mice (4, 21), and to induce mammary gland hyperplasia and carcinomas in transgenic mice when expressed under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (47). Alterations in cyclin E have similarly been documented in several human tumor types (15, 17). In particular, overexpression and alterations in cyclin E have been demonstrated in strong association with human breast cancer, with the severity of the alterations correlating with tumor stage and grade (6, 15, 17). Normal cell cycle regulation of cyclin E has also been shown to be defective in breast cancer cells, where cyclin E is expressed constitutively in an active complex with cdk2 throughout the cell cycle (16).

In this study, transgenic mice were utilized in an attempt to determine whether cyclin E plays a direct role in mammary tumorigenesis. Human cyclin E was expressed in transgenic mice under the control of the ovine β-lactoglobulin (BLG) promoter, which directs transgene expression to the mammary glands during pregnancy and lactation (11). A contributing role for cyclin E in mammary tumorigenesis was revealed by the cyclin E transgenic mice, which exhibited an induction of mammary gland hyperplasia and carcinomas.

MATERIALS AND METHODS

Generation and identification of transgenic mice. Transgene construct BLGCYCE was generated by cloning a 1.6-kb *Hin*dIII fragment containing the human cyclin E cDNA (18) into the *Eco*RV site of the ovine BLG promoter expression vector, pBJ41, as described previously for other transgene constructs (5). BLGCYCEINT was constructed by cloning the 300-bp *Eco*RV-*Xba*I fragment of pSL1190i (20) containing a generic intron into the *Eco*RV site of pBJ41 prior to insertion of the human cyclin E cDNA. The 8- and 8.3-kb transgene fragments of BLGCYCE and BLGCYCEINT, respectively, were released from vector sequences by digestion with *Sal*I and *Xba*I and were purified as previously described (5). Transgenic mice were generated by pronuclear microinjection of

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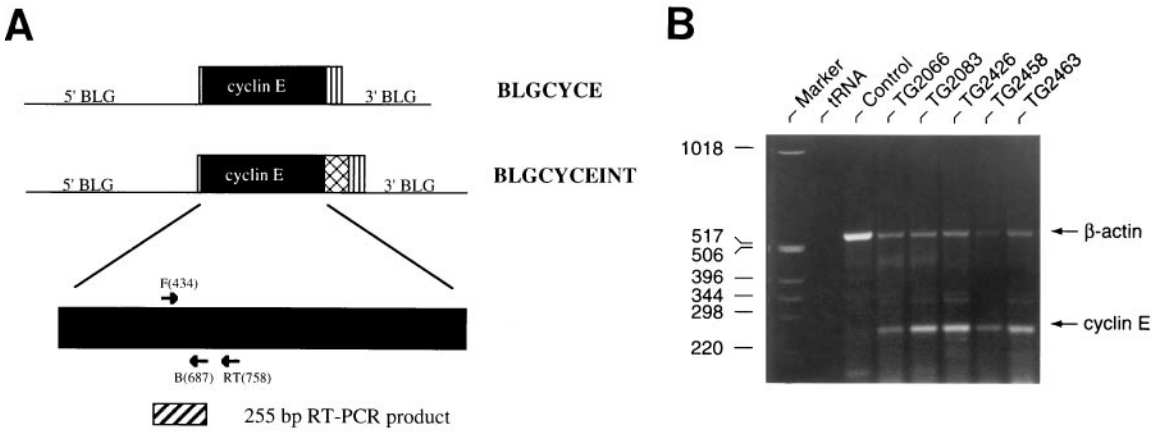

FIG. 1. Generation of transgenic mice expressing cyclin E in lactating mammary glands. (A) Transgene constructs. BLGCYCE contains the human cyclin E cDNA cloned into the BLG promoter expression vector, while BLGCYCEINT contains a 300-bp generic intron cloned 3′ of the cyclin E cDNA. Horizontal lines, BLG regulatory sequences; open boxes, BLG exons; black box, cyclin E cDNA; cross-hatched box, generic intron. The primers used for RT-PCR analysis which generated a 255-bp product are indicated by arrows. (B) RT-PCR analysis of transgene expression. RT-PCR was performed with primers specific to human cyclin E or to β-actin using 5 μg of total RNA prepared from midlactation mammary glands of the indicated lines of cyclin E transgenic and nontransgenic control mice. Arrows indicate the 540-bp β-actin and 255-bp cyclin E products.

B6C3F2 mouse embryos as described previously (33). Transgenic mice were identified by PCR analysis, with confirmation by Southern blotting. The following primers were used for PCR analysis: BLG, 5′-GCCACCCCGGGGCCTGAGG ATGAGC-3′; CYCE, 5′-GCTGGGGATACTGCGGCAGTAGCAC-3′; TSH5′, 5′-TCCTCAAAGATGCTCATTAG-3′; and TSH3′, 5′-GTAACTCACTCATG CAAAGT-3′. The BLG-CYCE primer set was used to detect BLGCYCE and BLGCYCEINT transgenes and produced an 857-bp product. The TSH5′-TSH3′ primer set was used as an internal control to amplify a 350-bp region of the thyroid-stimulating hormone gene. PCR conditions were as described previously (5). Verification of positive founders was done by Southern blotting with a 1.5-kb *Bam*HI-*Sph*I fragment of the BLG promoter as the probe.

Preparation and analysis of RNA. Total cellular RNA was prepared from mouse tissues as described previously (5). Reverse transcriptase (RT) PCR analysis was performed using the following primers specific to human cyclin E: RT(758), 5′-CAGGACACAATAGTC-3′; F(434), 5′-CAGCCAAAAATGCGA GCAATTC-3′; B(687), 5′-TTCATCTCCTGAACAAGCTCCATC-3′. RT-PCR was also performed with primers to amplify β-actin (CLONTECH Laboratories, Inc.) to serve as an internal control. For first-strand cDNA synthesis, 5 μg of RNA was combined with 2 μl of 10× buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin [Perkin-Elmer Cetus]), 2 μl of 0.1 mM deoxynucleotide triphosphate mixture, and 1 μl of RT primer (50 ng/μl) in a 19-μl reaction mixture; the mixture was heated to 70°C for 5 min and chilled on ice. One microliter of Superscript RT (Gibco BRL) was added, and the mixture was incubated for 10 min at room temperature and for 45 min at 45°C. For PCR amplification, 3 μl of 10× buffer, 1.3 μl of test or control primer sets (200 ng/μl each), and 1 μl of native *Taq* DNA polymerase (Perkin-Elmer Cetus) were combined with the cDNA mixture in a 50-μl reaction mixture, which was overlaid with a 20-μl bead of Paraplast Plus tissue embedding medium (Oxford Labware). Forty cycles of denaturation, annealing, and extension were performed in a Techne PHC-3 thermal cycler, with each cycle consisting of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C. Samples were subjected to electrophoresis through a 1.8% agarose–Tris-borate-EDTA gel and visualized by ethidium bromide staining. RT-PCR products of 540 and 255 bp were produced with the cyclin E and β-actin primer sets, respectively. Identical products were produced when RNA samples were treated with DNase I (10 μg of RNA, 5 mM $MgCl_2$, 0.5 μM dithiothreitol, 0.2 U of RNasin [Boehringer Mannheim] per μl, 0.025 U of DNase I [Boehringer Mannheim] per μl) for 20 min at 37°C; this was followed by phenol extraction, ethanol precipitation, and suspension in RNase-free distilled water prior to cDNA synthesis.

Histology and immunohistochemistry. Mammary glands and tumors obtained from animal biopsies and necropsies were fixed in Z-fix (Anatech), then embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. For immunohistochemical analysis, midlactation mammary glands were fixed in Bouin's fixative (Sigma) and embedded in paraffin and standard 5-μm sections were subjected to immunohistochemistry as previously described (5) by using rabbit polyclonal cyclin E antibodies (Upstate Biotechnology, Inc. [UBI]).

Preparation of protein extracts and Western blotting analysis. Protein extracts were prepared from frozen mouse tumor samples by homogenization in 2× radioimmunoprecipitation assay (RIPA) buffer (100 mM Tris-HCl [pH 7.5]), 300 mM NaCl, 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate [SDS], 2 mM sodium orthovanadate, 0.02 mg of aprotinin per ml, 0.1 mg of leupeptin per ml, 2 mg of Pefabloc per ml) followed by preclearing and protein concentration determination as described previously (5). For Western blotting analysis, 200 μg of each protein extract was subjected to electrophoresis through a 10% polyacrylamide-SDS gel; this was followed by transfer to nitrocellulose. Filters were blocked with 3% powdered milk in Tris-buffered saline–Tween 20 (0.2 M NaCl, 50 mM Tris-Cl [pH 7.4], 0.1% Tween 20) and incubated for 1 h with the appropriate primary antibody diluted in blocking solution and for 1 h with the appropriate secondary antibody diluted in blocking solution. Following each antibody incubation step, filters were washed three times in Tris-buffered saline–Tween 20. Detection was performed with the enhanced chemiluminescence detection system (Amersham). Primary antibodies were a mouse monoclonal antibody specific to human cyclin E (HE12; Santa Cruz Biotechnology) and a rabbit polyclonal antibody to cdk2 (UBI). The secondary antibodies were peroxidase-conjugated donkey anti-mouse immunoglobulin G and donkey anti-rabbit immunoglobulin G obtained from Jackson ImmunoResearch Laboratories, Inc.

Analysis of H1 kinase activity. To detect the presence of H1 kinase activity, 100 μg of each protein extract was combined with 5 μg of rabbit polyclonal cyclin E or cdk2 antibodies (UBI) in a 100-μl volume of 1× RIPA buffer. Samples were incubated for 16 h at 4°C with rotation; this was followed by the addition of 50 μl of protein A/G PLUS agarose (Santa Cruz Biotechnology) and incubation for 1 h at 4°C with rotation. Agarose beads were collected by microcentrifugation and washed once in 1× RIPA buffer, once in 1× RIPA buffer containing 1 M NaCl, and once in 50 mM HEPES (pH 7.5)–150 mM NaCl. The beads were suspended in 50 μl of H1 assay buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, 100 μg of histone H1 [Boehringer Mannheim] per ml, 1 mM dithiothreitol, 2 mM EGTA, 20 μM ATP, 200 μCi of [γ-^{32}P]ATP per ml) and incubated for 10 min at 30°C; this was followed by the addition of 50 μl of 2× SDS sample buffer (Novex) and electrophoresis through a 12.5% polyacrylamide–SDS gel. The gel was analyzed by autoradiography, and quantification was performed by phosphoimaging analysis (Molecular Dynamics).

Animal use. Glaxo Wellcome is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

RESULTS AND DISCUSSION

To determine the role of cyclin E in mammary tumorigenesis, transgenic mice were created which expressed human cyclin E in the mammary glands during pregnancy and lactation. Transgene constructs, depicted in Fig. 1A, consisted of the human cyclin E cDNA (18) either alone or in conjunction with a generic intron (BLGCYCE and BLGCYCEINT, respectively) under the control of the ovine BLG promoter (1, 48). The generic intronic sequence has been demonstrated to enhance heterologous gene expression in transgenic mice (7). Six transgenic founder animals were produced by pronuclear microinjection of the transgene constructs into B6C3F2 embryos (33). From these initial founders, five independent lines

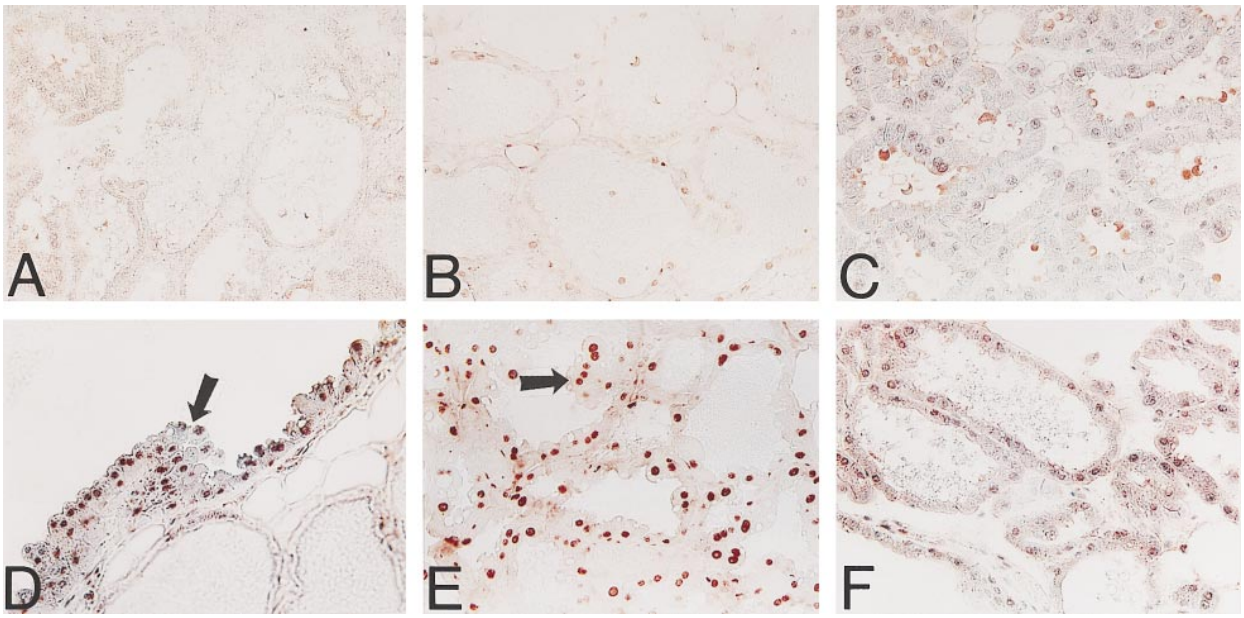

FIG. 2. Cyclin E protein expression in transgenic mammary glands. Immunohistochemistry was performed with polyclonal cyclin E antibodies on midlactation mammary gland sections from nontransgenic control mice (A) or cyclin E transgenic mice (B, TG2066 BLGCYCE; C, TG2083 BLGCYCE; D, TG2426 BLGCYCEINT; E, TG2458 BLGCYCEINT; F, TG2463 BLGCYCEINT). Red staining indicates positive reactivity. Arrows in panels D and E indicate papillary structures. Magnification, ×250.

of mice were established, two containing the BLGCYCE transgene (TG2066 and TG2083) and three containing the BLGCYCEINT construct (TG2426, TG2458, and TG2463). RT-PCR analysis of midlactation mammary gland samples indicated that cyclin E transgene expression occurred in each independent line of cyclin E transgenic mice (Fig. 1B). Transgene expression was not observed by RT-PCR in other tissues (data not shown), consistent with previous reports demonstrating that the BLG promoter confers specific expression to the mammary glands of pregnant and lactating transgenic mice (1, 5, 11, 40, 48).

Cyclin E expression in the transgenic mice was confirmed by immunohistochemical analysis of midlactation mammary gland sections. Immunoreactive cyclin E protein was present in the nuclei of mammary epithelial cells in BLGCYCE and BLGCYCEINT mammary glands, whereas endogenous cyclin E protein was not detected in midlactation mammary glands from nontransgenic animals (Fig. 2). Although not a precise quantitative measure of transgene expression, immunohistochemical staining for cyclin E protein was stronger in mammary glands of the BLGCYCEINT (Fig. 2D to F) transgenic mice than in those of the BLGCYCE (Fig. 2B and C) transgenic mice, possibly a result of increased expression due to the presence of the generic intron. Histopathological assessment revealed that lactating mammary glands of the cyclin E transgenic animals contained areas of hyperplasia relative to control animals (Fig. 3A to C). The hyperplasia was characterized primarily by papillary projections of hyperplastic cells, which stained strongly for cyclin E protein expression (Fig. 2). The occurrence of mammary gland hyperplasia during lactation in the cyclin E transgenic mice did not correlate with the number of pregnancies or cycles of cyclin E transgene expression, as hyperplastic projections were evident in mammary glands during the first lactation cycle (data not shown).

The hyperplastic structures in the cyclin E transgenic mammary glands did not routinely persist after weaning, when down-regulation of the BLG promoter occurs, but were generally eliminated during mammary gland regression. However, in approximately 10 to 15% of postbreeding cyclin E transgenic animals, regions of the mammary glands exhibited the histological appearance of lactation, including the papillary projections evident in lactating cyclin E transgenic mammary glands (Fig. 3D to F). The presence of mitotic figures was detected in these hyperplastic mammary glands (data not shown), possibly indicative of a preneoplastic state.

Neoplastic transformation has occurred in the cyclin E transgenic animals, with the development of mammary tumors in three independent lines of mice (Table 1). The tumors were classified by histopathological characterization as acinar adenocarcinomas with moderate to high mitotic indexes (Fig. 3G to I). In addition, the mammary adenocarcinomas were characterized by areas of moderate to extensive necrosis, suggesting rapid growth of the tumors. Mammary tumors have developed in approximately 12% of female cyclin E transgenic mice, with the age at tumor onset ranging from 8 to 13 months (Table 1). No incidence of mammary tumor formation has been observed in male cyclin E transgenic mice (data not shown) or in female nontransgenic littermate or inbred C57BL/6J control mice up to 18 months of age (Table 1). Although still under investigation, mammary tumor development in the cyclin E transgenic mice did not appear to correspond directly to the number of pregnancies. In one case a mammary adenocarcinoma developed in a female founder animal which had only a single litter and from which a line of mice could not be established (Table 1 and data not shown).

Tumors other than mammary adenocarcinomas have developed in the cyclin E transgenic mice, including adenomas and adenocarcinoma of the harderian gland and a lymphoma (Table 1). Although RT-PCR analysis did not reveal cyclin E transgene expression in normal tissues other than those of the mammary glands (data not shown), the sensitivity may not have been sufficient to detect the presence of rare transcripts that may have been produced by low-level or aberrant promoter activity in other tissues. The lymphoma isolated from a

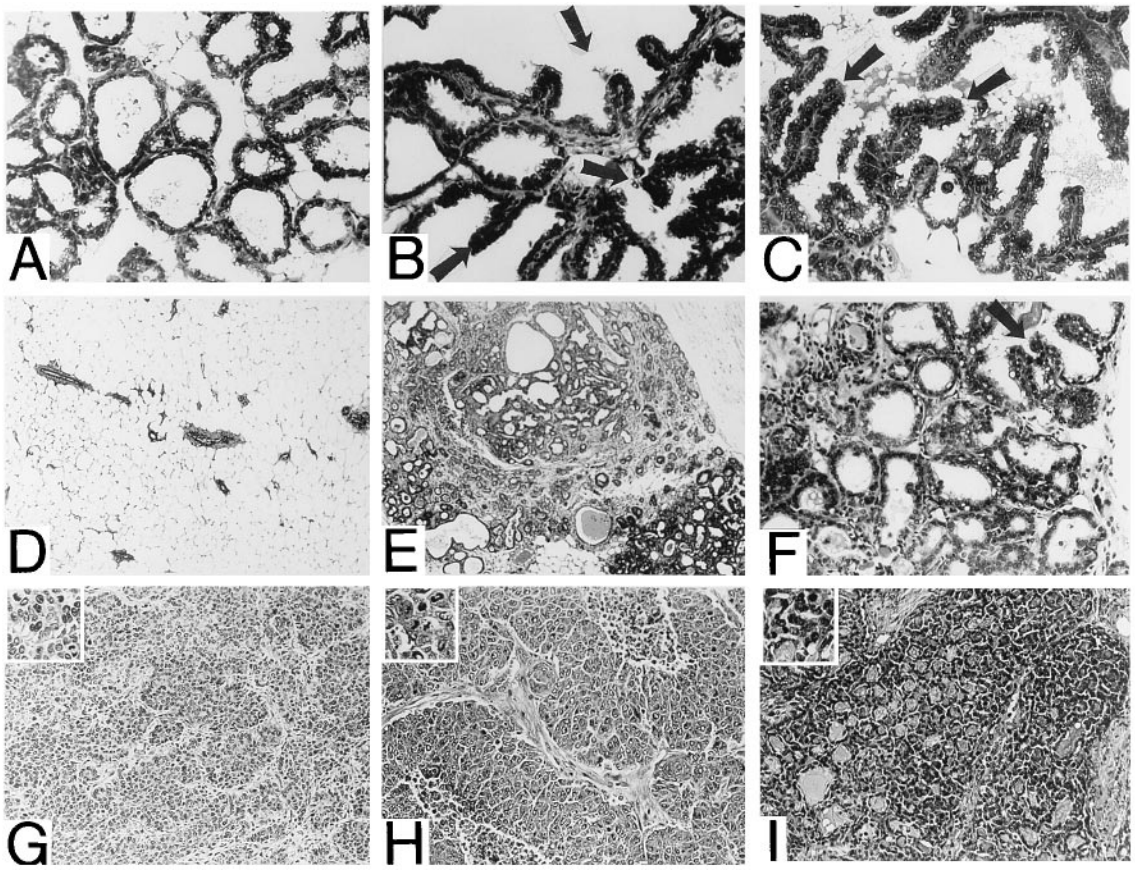

FIG. 3. Histology of cyclin E transgenic mammary glands and tumors. (A to C) Mammary glands from midlactation nontransgenic control (A) and cyclin E transgenic (B, TG2458 BLGCYCEINT; C, TG2463 BLGCYCEINT) animals stained with hematoxylin and eosin. Arrows indicate papillary projections of hyperplastic cells present in the transgenic glands. Magnification, ×250. (D to F) Mammary gland sections from age-matched (18-month-old) postbreeding nontransgenic control (D) and cyclin E transgenic (E and F, TG2463 BLGCYCEINT) animals stained with hematoxylin and eosin. Evident in panel F, which is a magnified view of the mammary gland section in panel E, are areas of hyperplasia (arrows) similar to those seen in lactating cyclin E transgenic mouse glands. Magnifications: D and E, ×250; F, ×500. (G to I) Mammary adenocarcinoma sections from three independent lines of cyclin E transgenic mice (G, TG2359 BLGCYCEINT; H, TG2458 BLGCYCEINT; I, TG2463 BLGCYCEINT) stained with hematoxylin and eosin. Magnifications: panels, ×250; insets, ×500.

cyclin E transgenic animal was demonstrated to contain transgene-specific cyclin E RNA (Fig. 4A), and expression of cyclin E protein was observed in both the lymphoma and harderian adenocarcinoma isolated from independent cyclin E transgenic animals (data not shown). These results suggest that cyclin E may have contributed to the development of other tumors in the transgenic mice, although the possibility that these tumors developed spontaneously solely because of age effects cannot be excluded.

RT-PCR analysis revealed the presence of transgene-specific cyclin E RNA in mammary tumors from the cyclin E transgenic mice, even though tumor formation occurred in postbreeding animals when the BLG promoter is not normally active (Fig. 4A). Western blotting analysis indicated that mammary tumors from the cyclin E transgenic mice contained proteins reactive with a human cyclin E-specific antibody, indicative of the presence of transgene-specific cyclin E protein in the tumors (Fig. 4B). Endogenous cdk2 protein was also detected in extracts from the cyclin E mammary tumors (Fig. 4B).

Functional assays were performed on tumor samples to determine whether the presence of cyclin E and cdk2 proteins reflected activity of the cyclin E-cdk2 kinase complex in the mammary tumors. Since cdk2 activity is regulated not only by cyclin association but also by stimulatory and inhibitory phosphorylation events (29, 43) and by association with small cdk inhibitory proteins (42), kinase assays were performed to determine whether cyclin E and cdk2 existed in an active complex in the tumor samples. Analysis of histone H1 kinase activity indicated that the mammary tumors and lymphoma from the

TABLE 1. Tumor incidence in female cyclin E transgenic mice

Mouse line	No. of mice[a]	No. with mammary tumors (mean age at onset [mo][b])	No. with other tumors[c] (mean age at onset [mo])	Total tumor incidence (%)
TG2066 BLGCYCE	8	0	0	0/8 (0)
TG2083 BLGCYCE	7	0	2 (14.0 ± 5.7)	2/7 (29)
TG2359 BLGCYCEINT	1	1 (13)	0	1/1 (100)
TG2426 BLGCYCEINT	3	0	1 (18)	1/3 (33)
TG2458 BLGCYCEINT	10	1 (10)	0	1/10 (10)
TG2463 BLGCYCEINT	11	3 (11.5 ± 3.7)	1 (16)	4/11 (36)
Nontransgenic control[d]	19	0	0	0/19 (0)

[a] Includes mice at least 11 months of age (mean age, 16 months), as well as mice in which tumors were detected at earlier ages.

[b] Mean age at which tumor (mammary carcinoma) was detected.

[c] Other tumor types include harderian adenomas (TG2083, TG2426), harderian adenocarcinoma (TG2083), and lymphoma (TG2463).

[d] Nontransgenic control mice include female nontransgenic littermate and C57BL/6J inbred mice at least 11 months of age (mean age, 15 months).

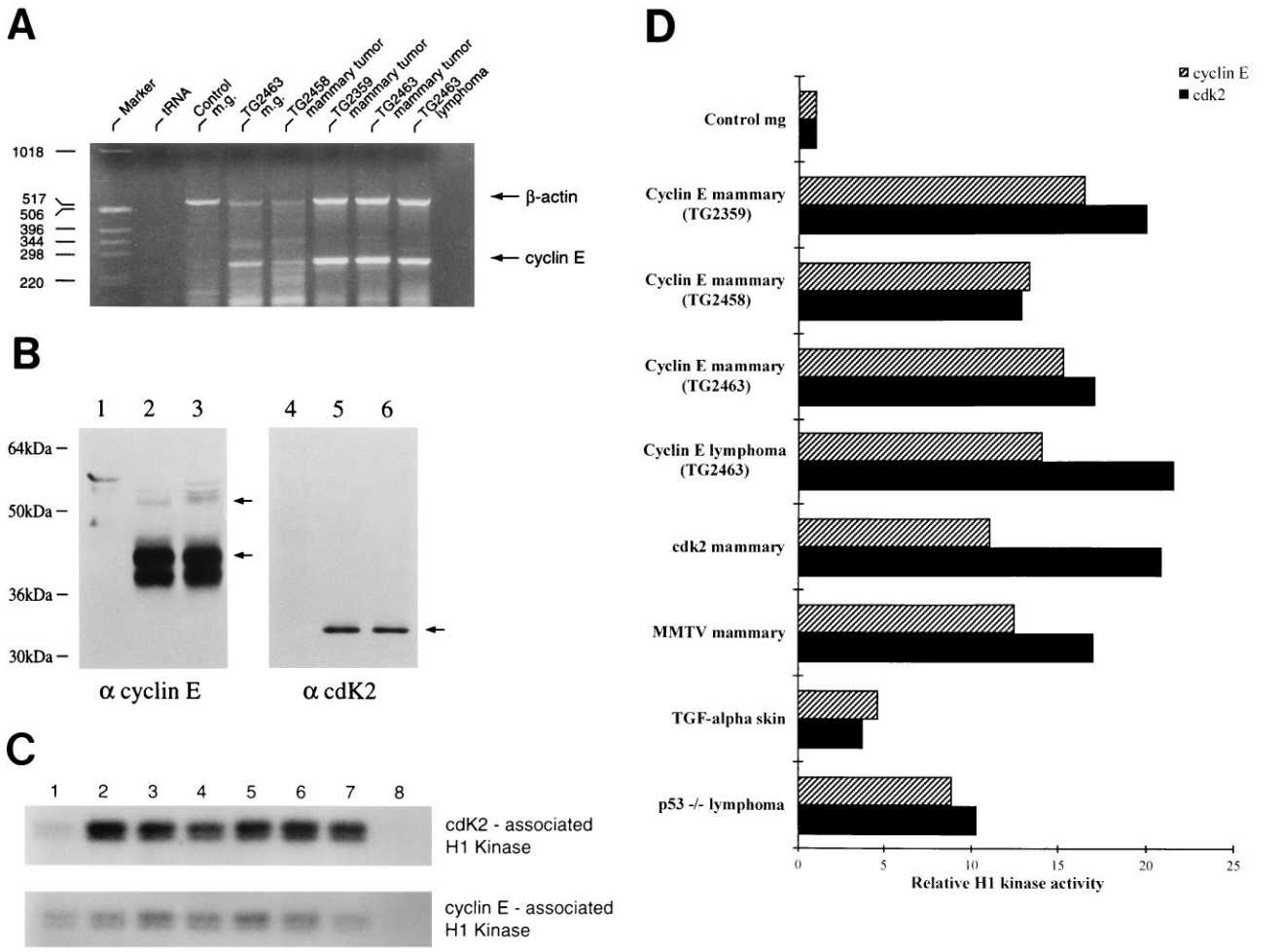

FIG. 4. Tumor analysis. (A) RT-PCR analysis of cyclin E transgene expression in tumor samples. RT-PCR was performed with cyclin E and β-actin primer sets on 5 μg of total RNA prepared from mammary glands (m.g.) of control and cyclin E transgenic mice and from the indicated cyclin E tumor samples. Arrows indicate the 540-bp β-actin and 255-bp cyclin E products. (B) Western blotting analysis of tumor extracts. Western blotting was performed with antibodies specific to human cyclin E (lanes 1 to 3) and to cdk2 (lanes 4 to 6). Each lane represents blotting performed with 200 μg of the following protein extracts: lanes 1 and 4, control lactating mammary gland; lanes 2 and 5, TG2359 BLGCYCEINT mammary tumor; lanes 3 and 6, TG2463 BLGCYCEINT mammary tumor. Arrows indicate the location of immunoreactive cyclin E proteins, which comigrated with reactive proteins in an epidermal growth factor-stimulated A431 cell extract (UBI) and in an extract containing baculovirus-produced human cyclin E (unpublished observations). The migration of protein standards (NOVEX SeeBlue) is indicated at the left. (C) Analysis of H1 kinase activity. Protein extracts (100 μg) were analyzed for the presence of histone H1 kinase activity using cdk2 or cyclin E antibodies to immunoprecipitate kinase complexes. Lanes: 1, transforming growth factor α skin tumor; 2, MMTV mammary tumor; 3, TG2359 BLGCYCEINT mammary tumor; 4, TG2458 BLGCYCEINT mammary tumor; 5, TG2463 BLGCYCEINT mammary tumor; 6, TG2463 BLGCYCEINT lymphoma; 7, TG1258 BLGCDK2 mammary tumor; 8, control mammary gland. (D) Relative histone H1 kinase activity in tumor samples. The cdk2- and cyclin E-associated kinase activities from several experiments were quantified by phosphoimaging analysis (Molecular Dynamics). The cdk2- and cyclin E-associated H1 kinase activities in the control mammary gland were arbitrarily designated 1 U each. The relative histone H1 kinase activities in the designated tumor samples were determined by comparison to the level of activity detected in the control mammary gland. Values are an average of three independent experiments, with the exception of the cdk2 mammary and cyclin E lymphoma samples, which are from one experiment. TGF, transforming growth factor.

cyclin E transgenic mice contained both functional cdk2- and cyclin E-associated kinase complexes and that the levels of kinase activity were significantly higher than that in a normal mammary gland (Fig. 4C). A quantification of relative kinase activity is shown in Fig. 4D, which indicates that cyclin E- and cdk2-associated kinase activities are elevated by approximately 13- to 22-fold in the cyclin E transgenic tumors relative to those in a normal mammary gland. Elevated levels of kinase activity were also detected in a mammary tumor that developed as a rare event in a transgenic animal expressing cdk2 under the control of the BLG promoter (5) and in a spontaneous mammary tumor taken from a C3H/HeOuJ-MMTV animal which harbors MMTV (Fig. 4C, D). Cyclin E- and cdk2-associated kinase activities were also detected at various levels in other murine tumors, including squamous cell carcinomas from transgenic mice expressing transforming growth factor α in the skin (20a) and lymphomas from p53-deficient animals (14) (Fig. 4D).

The presence of transgene-specific cyclin E RNA and protein in mammary tumors from the cyclin E transgenic mice, as well as the demonstration of functional cyclin E-cdk2 activity in the tumors, suggests that cyclin E is a contributing component of tumorigenic progression in this model system. Further support for a role for cyclin E in mammary tumor development comes from the occurrence of mammary tumors in three independent lines of cyclin E transgenic mice, whereas spontaneous tumor development has not been observed in nontransgenic animals of the same strain. In addition, endogenous

cyclin E-cdk2 activity is upregulated in mammary tumors that developed in MMTV-harboring and cdk2 transgenic mice, providing additional corroborative evidence for the involvement of cyclin E in mammary tumorigenesis.

Several studies have substantiated the association of cyclin E and D-type cyclins with human tumors, particularly breast cancer (6, 13, 15, 17). The present study provides evidence for the involvement of cyclin E in mammary tumor development, similar to previous studies with transgenic mice expressing cyclin D1 under the control of the MMTV long terminal repeat, which demonstrated that cyclin D1 may play a causative role in mammary tumorigenesis (47). Overexpression of either cyclin E or cyclin D1 in the mammary glands of mice results in proliferative disturbances, including hyperplastic lesions and tumor formation, although the timing of hyperplasia appearance is different in the two model systems, most likely due to differences in the promoters utilized. Whereas proliferative disturbances were first detected at 2 months of age in nulliparous cyclin D1 transgenic mice, mammary gland hyperplasia in cyclin E transgenic mice was primarily dependent upon pregnancy and lactation, when the BLG promoter is functional. The fairly low incidence of mammary tumors in the cyclin E transgenic mice may also be the result of promoter choice, because of the confinement of transgene expression by the BLG promoter to mammary epithelial cells during pregnancy and lactation and the resultant loss of these cells during involution. As a result, the majority of mammary epithelial cells which overexpress cyclin E and exhibit properties relevant to tumor development are effectively eliminated through the normal process of postlactational regression. Events which result in the retention or induction of cyclin E-expressing cells following regression, which have been detected in cyclin E transgenic mice, may be a necessary prerequisite for tumorigenesis. Mammary tumors in the cyclin E and cyclin D1 transgenic mice develop after similarly long latencies, suggesting a requirement in both systems for additional events in eliciting a tumorigenic response.

Consistent with the similarity in phenotypes observed in transgenic studies, both cyclin D1 and cyclin E have similar effects when overexpressed in cell lines, i.e., a shortened G_1 phase, reduced growth factor requirement, and decreased cell size (31, 36, 39, 49). However, several lines of evidence indicate that the specific events controlled by cyclins D1 and E are likely to be different. First, in cells stimulated to enter the cell cycle, cyclin D1 expression occurs earlier in G_1 phase than cyclin E appearance (9, 24, 30, 44). Second, although overexpression of cyclin D1 or E results in a similar reduction in the G_1 phase interval, expression of cyclin D1 leads to immediate Rb hyperphosphorylation, whereas cyclin E expression does not (38). Third, overexpression of both cyclin D1 and cyclin E in the same cell results in an additive effect on G_1 phase shortening, while the effect on Rb phosphorylation is similar to the effect seen with cyclin D1 alone (38). Fourth, while cyclin D1 is not required for progression from G_1 into S phase in Rb-deficient cells (23), cyclin E is essential for S phase entry regardless of Rb status (32). Therefore, cyclins D1 and E are likely to control different, complementary events that are necessary for G_1/S phase progression. Additional studies are under way to determine whether D-type cyclins and cyclin E also function in a cooperative manner in inducing neoplastic progression when expressed in the mammary glands of transgenic mice.

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REFERENCES

1. **Archibald, A. L., M. McClenaghan, V. Hornsey, J. P. Simons, and A. J. Clark.** 1990. High-level expression of biologically active human 1-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci. USA **87:**5178–5182.
2. **Baldin, V., J. Lukas, M. J. Marcote, M. Pagano, and G. Draetta.** 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. **7:**812–821.
3. **Bates, S., L. Bonetta, D. MacAllan, D. Parry, A. Holder, C. Dickson, and G. Peters.** 1994. Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. Oncogene **9:**71–79.
4. **Bodrug, S. E., B. J. Warner, M. L. Bath, G. L. Lindeman, A. W. Harris, and J. W. Adams.** 1994. Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the *myc* gene. EMBO J. **13:**2124–2130.
5. **Bortner, D. M., and M. P. Rosenberg.** 1995. Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis. Cell Growth Differ. **6:**1579–1589.
6. **Buckley, M. F., K. J. E. Sweeney, J. A. Hamilton, R. L. Sini, D. L. Manning, R. I. Nicholson, A. deFazio, C. K. W. Watts, E. A. Musgrove, and R. I. Sutherland.** 1993. Expression and amplification of cyclin genes in human breast cancer. Oncogene **8:**2127–2133.
7. **Choi, T., M. Huang, C. Gorman, and R. Jaenisch.** 1991. A generic intron increases gene expression in transgenic mice. Mol. Cell. Biol. **11:**3070–3074.
8. **Draetta, G.** 1994. Mammalian G1 cyclins. Curr. Opin. Cell Biol. **6:**842–846.
9. **Dulic, V., L. Drullinger, E. Lees, S. Reed, and G. Stein.** 1993. Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-cdk2 and cyclin D1-cdk2 complexes. Proc. Natl. Acad. Sci. USA **90:**11034–11038.
10. **Dulic, V., E. Lees, and S. I. Reed.** 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. Science **257:**1958–1961.
11. **Harris, S., M. McClenaghan, J. P. Simons, S. Ali, and A. J. Clark.** 1991. Developmental regulation of the sheep β-lactoglobulin gene in the mammary gland of transgenic mice. Dev. Genet. **12:**299–307.
12. **Hinds, P. W., S. F. Dowdy, E. N. Eaton, A. Arnold, and R. A. Weinberg.** 1994. Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. USA **91:**709–713.
13. **Hunter, T., and J. Pines.** 1994. Cyclins and cancer. II. Cyclin D and cdk inhibitors come of age. Cell **79:**573–582.
14. **Jacks, T., L. Remington, B. Williams, E. Scmitt, S. Halachmi, R. Bronson, and R. Weinberg.** 1994. Tumor spectrum analysis in p53-mutant mice. Curr. Biol. **4:**1–7.
15. **Keyomarsi, K., and A. B. Pardee.** 1993. Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc. Natl. Acad. Sci. USA **90:** 1112–1116.
16. **Keyomarsi, K., D. Conte, W. Toyofuku, and M. P. Fox.** 1995. Deregulation of cyclin E in breast cancer. Oncogene **11:**941–950.
17. **Keyomarsi, K., N. O'Leary, G. Molnar, E. Lees, H. J. Fingert, and A. B. Pardee.** 1994. Cyclin E, a potential prognostic marker for breast cancer. Cancer Res. **54:**380–385.
18. **Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. M. Roberts.** 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell **66:**1217–1228.
19. **Koff, A., A. Giordano, D. Desai, K. Yamashita, W. Harper, S. Elledge, T. Nishimoto, D. Morgan, R. Franza, and J. Roberts.** 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689–1693.
20. **Kucera, G. T., D. M. Bortner, and M. P. Rosenberg.** 1996. Overexpression of an agouti cDNA in the skin of transgenic mice recapitulates dominant coat color phenotypes of spontaneous mutants. Dev. Biol. **173:**162–173.

20a. **Kucera, G. T., D. Hawkins-Brown, and M. P. Rosenberg.** Unpublished data.

21. **Lovec, H., A. Grzeschiczek, and T. Moroy.** 1994. Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphomas in transgenic mice. EMBO J. **13:**3487–3495.
22. **Lovec, H., A. Sewing, F. C. Lucibello, R. Muller, and T. Moroy.** 1994. Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene **9:**323–326.
23. **Lukas, J., J. Bartkova, M. Rohde, M. Strauss, and J. Bartek.** 1995. Cyclin D1 is dispensable for G_1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. **15:**2600–2611.
24. **Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek.** 1994. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumor cell lines. Oncogene **9:**707–718.
25. **Matsushime, H., M. E. Ewen, D. K. Strom, J. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr.** 1992. Identification and properties of an atypical subunit $p34^{PSK\text{-}J3}$ for mammalian D-type G1 cyclins. Cell **71:**323–334.
26. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and**

J.-Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. **14:**2066–2076.
27. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077–2086.
28. **Meyerson, M., G. H. Enders, C. Wu, L. Su, C. Gorka, C. Nelson, E. Harlow, and L. Tsai.** 1992. A family of human cdc2-related protein kinases. EMBO J. **11:**2909–2917.
29. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature **374:**131–134.
30. **Musgrove, E. A., J. A. Hamilton, C. S. L. Lee, K. J. E. Sweeney, C. K. W. Watts, and R. I. Sutherland.** 1993. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. Mol. Cell. Biol. **13:**3577–3587.
31. **Ohtsubo, M., and J. Roberts.** 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science **259:**1908–1912.
32. **Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano.** 1995. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15:**2612–2624.
33. **Osborn, L., M. P. Rosenberg, S. A. Keller, and M. H. Meisler.** 1987. Tissue-specific and insulin-dependent expression of a pancreatic amylase gene in transgenic mice. Mol. Cell. Biol. **7:**326–334.
34. **Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek, and G. Draetta.** 1993. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. J. Cell Biol. **121:**101–111.
35. **Pines, J.** 1993. Cyclins and cyclin-dependent kinases: take your partners. Trends Biochem. Sci. **18:**195–197.
36. **Quelle, D., R. Ashmun, S. Shurtleff, J. Kato, D. Bar-Sagi, M. Roussel, and C. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
37. **Reed, S. I.** 1992. The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol. **8:**529–561.
38. **Resnitzky, D., and S. I. Reed.** 1995. Different roles for cyclins D1 and E in regulation of the G_1-to-S transition. Mol. Cell. Biol. **15:**3463–3469.
39. **Resnitzky, D., M. Gossen, H. Bujard, and S. I. Reed.** 1994. Acceleration of the G_1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol. Cell. Biol. **14:**1669–1679.
40. **Shani, M., I. Barash, M. Nathan, G. Ricca, G. H. Searfoss, I. Dekel, A. Faerman, D. Givol, and D. R. Hurwitz.** 1992. Expression of human serum albumin in the milk of transgenic mice. Transgenic Res. **1:**195–208.
41. **Sherr, C.** 1994. G1 phase progression: cycling on cue. Cell **79:**551–555.
42. **Sherr, C. J., and J. M. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9:**1149–1163.
43. **Solomon, M. J.** 1994. The function(s) of CAK, the p34cdc2-activating kinase. Trends Biochem. Sci. **19:**494–500.
44. **Tam, S. W., A. M. Theodoras, J. W. Shay, G. F. Draetta, and M. Pagano.** 1994. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with cdk4 is required for cyclin D1 function in G1 progression. Oncogene **9:**2663–2674.
45. **Tsai, L.-H., E. Lees, B. Faha, E. Harlow, and K. Riabowol.** 1993. The cdk2 kinase is required for the G1 to S transition in mammalian cells. Oncogene **8:**1593–1602.
46. **van den Heuvel, S., and E. Harlow.** 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science **262:**2050–2054.
47. **Wang, T. C., R. D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E. V. Schmidt.** 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature **369:**669–671.
48. **Whitelaw, C. B. A., A. L. Archibald, S. Harris, M. McClenaghan, J. P. Simons, and A. J. Clark.** 1991. Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. Transgenic Res. **1:**3–13.
49. **Wimmel, A., F. Lucibello, A. Sewing, S. Adolph, and R. Muller.** 1994. Inducible acceleration of G1 progression through tetracyclin-regulated expression of human cyclin E. Oncogene **9:**995–997.
50. **Xiong, Y., H. Zhang, and D. Beach.** 1992. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell **71:**504–514.