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Growth Factor, Steroid, and Steroid Antagonist Regulation of Cyclin Gene Expression Associated with Changes in T-47D Human Breast Cancer Cell Cycle Progression

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Cyclins and proto-oncogenes including c-*myc* have been implicated in eukaryotic cell cycle control. The role of cyclins in steroidal regulation of cell proliferation is unknown, but a role for c-*myc* has been suggested. This study investigated the relationship between regulation of T-47D breast cancer cell cycle progression, particularly by steroids and their antagonists, and changes in the levels of expression of these genes. Sequential induction of cyclins D1 (early G_1 phase), D3, E, A (late G_1-early S phase), and B1 (G_2 phase) was observed following insulin stimulation of cell cycle progression in serum-free medium. Transient acceleration of G_1-phase cells by progestin was also accompanied by rapid induction of cyclin D1, apparent within 2 h. This early induction of cyclin D1 and the ability of delayed administration of antiprogestin to antagonize progestin-induced increases in both cyclin D1 mRNA and the proportion of cells in S phase support a central role for cyclin D1 in mediating the mitogenic response in T-47D cells. Compatible with this hypothesis, antiestrogen treatment reduced the expression of cyclin D1 ~8 h before changes in cell cycle phase distribution accompanying growth inhibition. In the absence of progestin, antiprogestin treatment inhibited T-47D cell cycle progression but in contrast did not decrease cyclin D1 expression. Thus, changes in cyclin D1 gene expression are often, but not invariably, associated with changes in the rate of T-47D breast cancer cell cycle progression. However, both antiestrogen and antiprogestin depleted c-*myc* mRNA by >80% within 2 h. These data suggest the involvement of both cyclin D1 and c-*myc* in the steroidal control of breast cancer cell cycle progression.

Steroid hormones have well-documented effects on proliferation in responsive tissues, including the breast and uterus, and tumors arising from these tissues often remain steroid responsive. The regulation of cellular proliferation by steroids and steroid antagonists occurs by cell cycle-specific actions on cells in G_1 phase (reviewed in reference 40). Estrogen stimulates breast cancer cell cycle progression (28, 29) at a point in early G_1 phase (26). Similarly, sensitivity to the growth-inhibitory effects of antiestrogens is restricted to a limited time frame in early G_1 phase (43, 60). Progestins both stimulate and inhibit cell cycle progression in breast cancer cells. The initial response is acceleration of cells in G_1 phase, apparently due to stimulation of a rate-limiting step in cell cycle progression, but following completion of a cell cycle, progestin-treated cells are arrested in G_1 phase (39, 58). Antiprogestins arrest cells in G_1 phase with kinetics similar to those of growth arrest by antiestrogens (42).

The central mechanism of steroid hormone action is the regulation of transcription by ligand-activated steroid hormone receptors binding to specific response elements in the regulatory regions of target genes (4). Steroid antagonists bind to the ligand-binding domain of steroid hormone receptors and interfere with their transactivation of gene expression (12, 67). Steroid and steroid antagonist effects on proliferation are thus likely to be mediated by modulation of the expression of a specific set of genes intimately involved in the control of cell cycle progression. However, the target genes responsible are unknown, at least in part because only a limited number of genes with potential cell cycle regulatory roles have been studied with hormone-responsive cells.

Recent investigation has identified cyclins and cyclin-dependent kinases (CDKs) as key regulators of cell cycle progression in eukaryotic cells. These molecules form the regulatory subunits (cyclins) and catalytic subunits (CDKs) of cell cycle-regulated kinases. Thus far, the best understood cyclin/CDK complexes are involved in the control of mitosis. Studies with yeasts and with amphibian and invertebrate oocytes showed that accumulation and subsequent destruction of cyclin B together with regulation of the activity of the associated $p34^{cdc2}$ kinase provides a mechanism for control of transit through mitosis (45). This mechanism has been widely conserved: functional homologs of cyclin B, $p34^{cdc2}$, and many elements of the network which regulates their activity have been identified in organisms as diverse as yeasts and mammals (45). Mammalian cells contain at least five cyclin classes (cyclins A to E), which reach maximum abundance at different points in the cell cycle (17). Similarly, at least four CDKs, *cdc2*, *cdk2*, *cdk3*, and *cdk4*, have been identified (11, 25, 31, 34, 44, 62). The transcriptional activation of cyclin genes and consequent transient accumulation of different cyclin proteins which then bind to one of the CDKs to initiate phosphorylation cascades are thought to be the central mechanism for a series of control points in the mammalian cell cycle (37, 45, 48, 53). Support for this view is provided by microinjection studies with anti-cyclin A antibodies, which have shown cyclin A to be necessary for progression through S phase (14, 46), and other studies showing that the *cdc2* kinase is necessary for mitosis (51).

In budding yeast, the G_1-to-S-phase transition requires

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distinct G_1 cyclins (CLN1, CLN2, and CLN3), which appear to be rate limiting for progress through G_1, since overexpression or expression of mutant cyclins encoding a stabilized protein reduces the duration of G_1 phase (references 48 and 53 and references therein). During G_1 as well as G_2 it appears that much of the basic mechanism for cell cycle control has been widely conserved, although it is clear that the regulation of the mammalian cell cycle is considerably more complex than cell cycle regulation in yeast. In synchronized or growth factor-stimulated mammalian cells, cyclins C, D1, D2, D3, and E are most abundant during G_1 phase (27, 32, 35, 36, 69), suggesting that these cyclins may function at G_1 control points in mammalian cells. This is supported by recent evidence that the kinase activity associated with cyclin E is maximal during late G_1-early S phase (9, 21) and that complex formation between D-type cyclins and the *cdk4* kinase is maximum in late G_1 phase (31). The yeast CLN1 and CLN2 proteins are unstable, and their abundance is largely transcriptionally regulated (48, 53). Cyclins C, D1, D2, D3, and E contain sequences which are thought to target proteins for rapid degradation (20, 27, 32, 36), while cyclin D1 and D2 mRNA and protein have short half-lives (31, 32), suggesting that the abundance and, in turn, the function of mammalian G_1 cyclins as well as yeast G_1 cyclins are primarily transcriptionally regulated.

We postulated that regulation of proliferation by steroids and steroid antagonists is associated with regulation of the expression of G_1 cyclins, since these agents have G_1-specific effects and act via receptor-mediated transcriptional activation. The primary aim of this study was to examine this possibility, using the well-characterized human breast cancer cell line T-47D. These cells are responsive to a range of growth factors as well as to estrogens, progestins, and their antagonists. In addition, none of the cyclin genes examined were either amplified or overexpressed in this cell line (5). Previous publications have suggested a role for c-*myc* in the regulation of proliferation by steroids (8, 39, 66), and therefore, the relationship between changes in the expression of this proto-oncogene and G_1 cyclins was also examined. Cellular responses to steroids and steroid antagonists can be modulated by growth factors often present in fetal calf serum (FCS) (22, 23, 55, 63). These interactions are complex, and the experiments in this study used a defined, serum-free culture system to more readily enable the effects of individual compounds to be studied.

MATERIALS AND METHODS

Reagents. Recombinant human insulin-like growth factor-I (IGF-I) was obtained from Boehringer Mannheim, Castle Hill, New South Wales, Australia; porcine insulin was generously donated by John Miller (CSL-Novo, North Rocks, New South Wales, Australia). The synthetic progestin ORG 2058 and steroid antagonists were obtained from the indicated sources: ORG 2058 (16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione), Amersham Australia, North Ryde, New South Wales, Australia; RU 486 [17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one], Jean-Pierre Raynaud of Roussel-Uclaf, Romainville, France; ICI 164384 [*N*-*n*-butyl-*N*-methyl-11-(3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl) undecanamide], Alan Wakeling, ICI Pharmaceuticals, Alderley Park, Cheshire, United Kingdom. ORG 2058, RU 486, and ICI 164384 were stored at −20°C as 1,000- or 2,000-fold-concentrated stock solutions in absolute ethanol. Other reagents were obtained from standard commercial suppliers.

Human cyclin and cyclin-dependent kinase cDNAs were generously provided by the indicated investigators: cyclin A, cyclin B1, and *cdk2*, Jonathon Pines and Tony Hunter (Salk Institute, San Diego, Calif. [49, 50]); cyclins C and E, Steven Reed (Scripps Research Institute, La Jolla, Calif. [27]); cyclins D1 and D3, Yue Xiong and David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. [71, 72]); *cdc2*, Paul Nurse (Imperial Cancer Research Fund Cell Cycle Group, University of Oxford, Oxford, United Kingdom [25]). A human histone H4 genomic clone was generously provided by Janet Stein (Department of Cell Biology, University of Massachusetts, Worcester, Mass. [47]). Either restriction enzyme digestion or polymerase chain reaction was used to obtain fragments for labelling, which encompassed the entire open reading frame except for cyclin D3 (approximately 60% of the open reading frame) and histone H4 (a 1.4-kbp fragment including 236 bp of 5′ untranslated region).

Cell culture. Stock cultures of T-47D cells (19), obtained at passage 74 from E.G. and G. Mason Research Institute, Worcester, Mass., and cryopreserved by standard techniques (59), were maintained as previously described but without antibiotics, in RPMI 1640 medium supplemented with 10 μg of human insulin (Actrapid; CSL-Novo) per ml and 10% FCS (58). The cell culture medium used in experiments contained phenol red-free RPMI 1640, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (20 mM), sodium bicarbonate (14 mM), L-glutamine (6 mM), and 20 μg of gentamicin per ml. Serum-free medium was supplemented with 300 nM human transferrin (Sigma Chemical Co., St. Louis, Mo.). Tissue culture experiments were performed essentially as described previously (39). Briefly, T-47D cells were taken from stock cultures and passaged for 6 days in phenol red-free medium containing 10% charcoal-treated FCS (52). During this time the cells received two changes of medium at 1- to 3-day intervals to deplete them of steroids. The cells were then replated into replicate flasks in medium containing 10 or 15% charcoal-treated FCS, and the medium was replaced with fresh serum-free medium on the next two days. In some experiments the final serum-free medium contained 10 μg (1.7 μM) of porcine insulin per ml. Three or more days after the completion of these pretreatments, growth factor, steroid, or steroid antagonist was added. Control flasks received vehicle to the same final concentration. Cell cycle phase distribution was determined by analytical DNA flow cytometry, as previously described (39, 43).

RNA isolation and northern (RNA) analysis. Cells harvested from triplicate flasks were pooled, RNA was extracted by a guanidinium-isothiocyanate-cesium chloride procedure, and Northern analysis was performed as previously described, with 10 to 20 μg of total RNA per lane (1, 39). The membranes were hybridized (50°C, overnight) with probes labelled with [α-^{32}P]dCTP (Amersham; specific activity, ~3,000 Ci/mmol) to a specific activity of 0.3×10^9 to 2×10^9 cpm/μg of DNA by using the Multiprime DNA labelling kit (Amersham). The membranes were washed at a highest stringency of 0.2× SSC (30 mM NaCl, 3 mM sodium citrate [pH 7.0])–1% sodium dodecyl sulfate at 65°C and exposed to Kodak X-OMAT film at −70°C. The mRNA abundance was quantitated by densitometric analysis of autoradiographs using a Bio-Rad Video Densitometer, model 620, and Bio-Rad 1-D Analyst software. The accuracy of RNA loading was estimated as previously described (39) by hybridizing membranes with a [γ-^{32}P]ATP end-labelled oligonucleotide complementary to 18S rRNA (6) and typi-

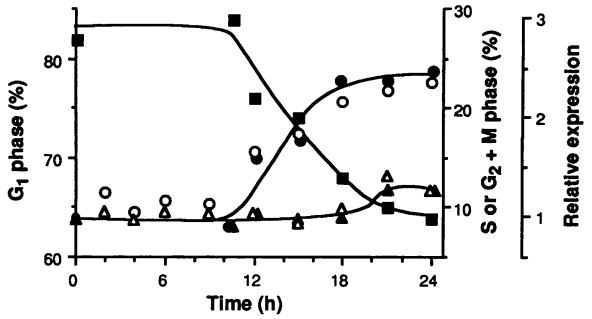

FIG. 1. Effect of insulin on T-47D cell cycle phase distribution, histone H4 expression, and cyclin B1 expression. T-47D cells maintained in serum-free medium were treated with 1.7 μM insulin and at intervals thereafter were harvested for DNA analysis by flow cytometry or RNA extraction in separate experiments. Cell cycle phase distributions were estimated by computer fit. Relative mRNA levels were estimated by densitometry and are presented relative to the average for seven control samples harvested throughout the experiment. ■, G_1 phase; ●, S phase; ▲, G_2 plus M phase; ○, histone H4; △, cyclin B1.

cally varied by ≤15% from the average value. Densitometric values are uncorrected for variations within this range.

RESULTS

Effects of insulin on cyclin and cyclin-dependent kinase expression. In unsupplemented serum-free medium (SF), T-47D cells were growth arrested; the cell number increased by less than 30% over 3 to 4 days. Addition of insulin (1.7 μM) induced a cohort of cells to reenter the cell cycle (41). By 12 h after insulin stimulation, the proportion of cells in S phase (%S phase) began to increase, reaching a plateau at 21 to 24 h; simultaneously, the proportion of cells in G_1 began to decrease (Fig. 1). The stimulated cells subsequently completed DNA replication (evidenced by increases in the proportion of cells in G_2 plus M [Fig. 1]) and divided (evidenced by increases in cell number [41]). The time course of changes in cell cycle phase distribution for insulin-stimulated cells was almost identical to that observed after mitotic selection of T-47D cells (unpublished data). Thus, insulin stimulation of serum-deprived cells provides a means of examining the relationship between cell cycle position and cyclin gene expression in breast cancer cells.

Histone H4 expression remained constant for 12 h after insulin treatment of cells maintained in serum-free medium but then increased to reach a maximum at 18 to 24 h (Fig. 1 and 2). Cyclin B1 expression remained unchanged until 21 h after insulin treatment but then increased (Fig. 1 and 2). These changes closely paralleled the changes in cell cycle phase distribution (Fig. 1). Thus, regulation of histone H4 and cyclin B1 gene expression allowed progress through S and G_2 phases, respectively, to be monitored, as expected from previous data showing that the expression of the histone H4 gene is tightly linked with progress through S phase (57) while cyclin B expression is linked with progress through G_2 phase into mitosis (49).

In unsupplemented serum-free medium, growth-arrested T-47D cells expressed readily detectable cyclin A (~2.7 kb), *cdc2* (~2.0 and 1.6 kb), and *cdk2* (~2.1 kb) mRNA. Following insulin stimulation, the expression of these genes remained constant until approximately 12 h, increased as the cells began to enter S phase, and at 21 h reached levels approximately two- to threefold greater than levels in untreated or vehicle-treated control cells (Fig. 2). Regulation of G_1 cyclin gene expression in insulin-stimulated cells was also examined. Cyclin D2 mRNA was present at barely detectable levels in exponentially growing cells (5) and was not further investigated. However, like cyclins A and B1, cyclins C (2.3 kb), D1 (4.5 and 1.5 kb), D3 (2.7 kb), and E (~2 kb) were detectable in growth-arrested T-47D cells (Fig. 3). Cyclin D1 mRNA levels increased sharply (~2.5-fold) within 2 h of insulin treatment, were maintained at this level until 12 h, and then began to decline slowly as cells entered S phase, returning almost to control levels by 24 h as the cells passed through G_2 phase (Fig. 3). Cyclin D3 mRNA increased more slowly, reaching a maximum at 15 h and remaining elevated as cells passed through S phase. Slight increases in the expression of cyclin E occurred within 2 h after insulin treatment, but the major increase began at 12 h, as the cells entered S phase, i.e., several hours later than the increases in cyclin D1 and D3 mRNA (Fig. 3). A maximal twofold induction was reached at approximately 18 h. The expression of cyclin E then declined as the majority of cells passed through S phase. In contrast with the other cyclins examined, no detectable changes were observed in cyclin C expression throughout the 24 h following insulin treatment (Fig. 3). In other experiments, slight changes in cyclin C expression were detected (<50% increase [data not shown]), but this was not a consistent observation.

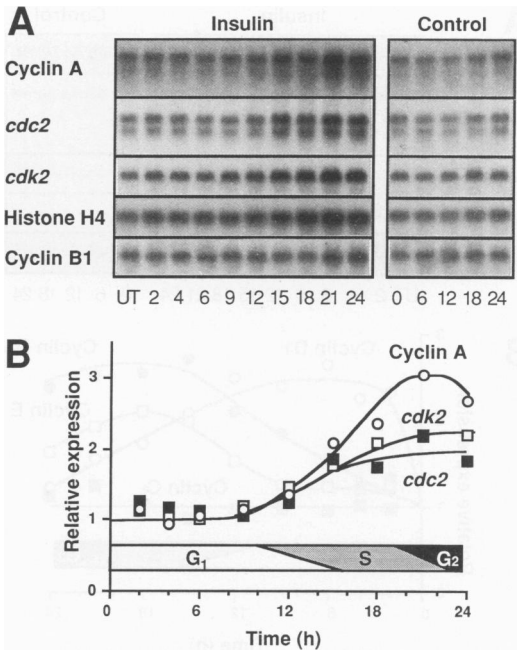

FIG. 2. Effect of insulin on the expression of cyclin A, *cdc2*, *cdk2*, histone H4, and cyclin B1. T-47D cells maintained in serum-free medium were treated with 1.7 μM insulin or vehicle control or were untreated (UT). Triplicate flasks were harvested at the indicated times. RNA was extracted for Northern analysis, and replicate filters containing 15 μg of total RNA per lane were probed for each mRNA species. Points in panel B were obtained by densitometric analysis of autoradiographs showing cyclin A, *cdc2*, and *cdk2* expression. The data are presented relative to the average for seven untreated or vehicle-treated control samples harvested over the duration of the experiment.

Relationship between G_1 cyclin induction and entry into S phase after mitogen stimulation. To establish whether the sequential induction of cyclin D1 and then cyclin D3 and E

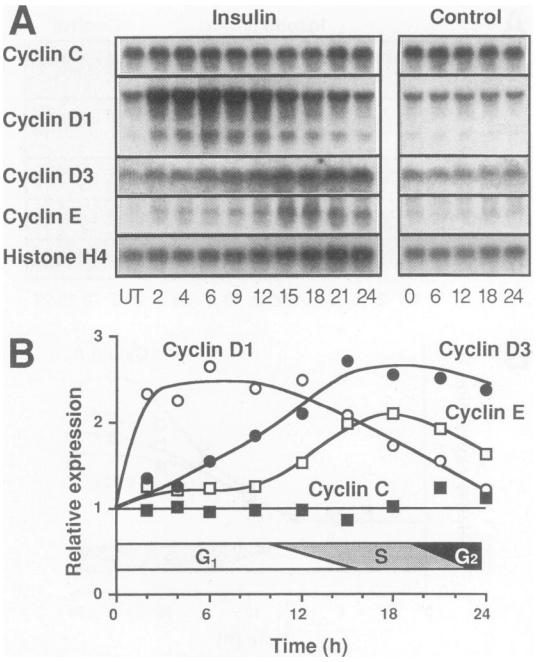

FIG. 3. Effect of insulin on the expression of cyclins C, D1, D3, and E. Replicate filters containing 15 μg of total RNA per lane from the experiment described for Fig. 2 were probed for each mRNA species (UT, untreated). Points in panel B were obtained by densitometric analysis of autoradiographs showing cyclin C, D1, D3, and E expression. The data are presented relative to the average for seven untreated or vehicle-treated control samples harvested over the duration of the experiment.

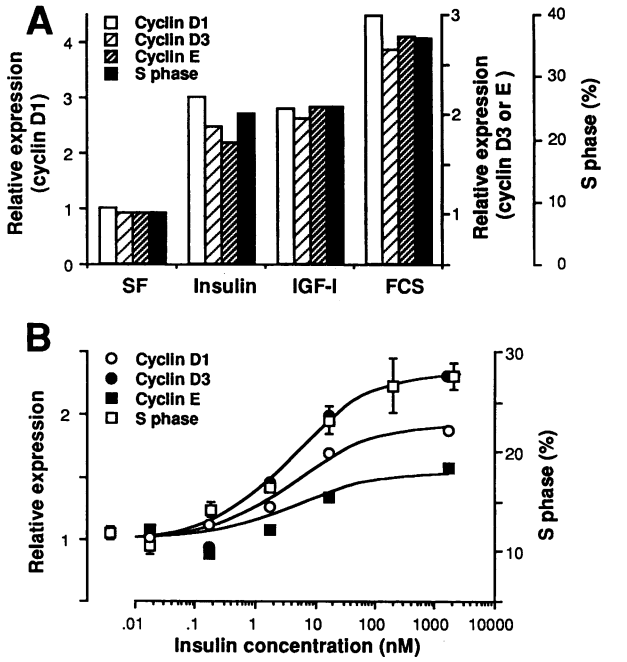

FIG. 4. Effect of insulin, IGF-I, and FCS on the expression of cyclins D1, D3, and E and entry into S phase. (A) T-47D cells maintained in serum-free medium were treated with vehicle (SF), insulin (1.7 μM), IGF-I (1 nM), or FCS (final concentration, 10%). Triplicate flasks were harvested, and RNA was extracted for Northern analysis at 6 h (cyclin D1) or 18 h (cyclin D3 and E). %S phase after 24 h of treatment in a representative experiment is also shown. (B) T-47D cells maintained in serum-free medium were treated with the indicated concentrations of insulin. Triplicate flasks were harvested for each concentration at 6 h (cyclin D1) or 18 h (cyclins E and D3). The data have been normalized to the averages for untreated and vehicle control samples harvested at each time and, for cyclin D3, corrected for variations in loading. Data for %S phase represent the means ± standard errors of the mean for three to five histograms from cells treated for 21 or 24 h in two independent experiments.

mRNA expression was characteristic of mitogens other than insulin, the effects of insulin, IGF-I, and FCS were compared after 6 and 18 h. All three treatments induced cyclin D1, D3, and E mRNA expression (~2-fold or more) (Fig. 4A). The increase in cyclin D1 mRNA levels at 6 h was not sustained at the later time, while conversely, increased cyclin D3 and E mRNA levels were observed at 18 h but not at 6 h. In each case, FCS was more effective than either insulin or IGF-I. The rank order observed for cyclin induction (insulin ≈ IGF-I < FCS) is the same as the rank order for relative proportion of cells entering S phase after these treatments (Fig. 4A) (41).

The relationship between the degree of induction of cyclins D1, D3, and E and the proportion of cells which later entered S phase was further examined, since the abundance of these cyclins may regulate transit through G_1 phase. Growth-arrested cells were treated with a range of insulin concentrations (17 pM to 1.7 μM) and harvested for Northern analysis at 6 or 18 h, when expression of cyclins D1 or D3 and E, respectively, was maximal. A concentration-dependent increase in the level of cyclin D1 and D3 mRNA was observed at insulin concentrations between 1.7 nM and 1.7 μM, while cyclin E expression increased at concentrations of ≥17 nM (Fig. 4B). The concentration dependence of the increases in the expression of these cyclins was in agreement with the concentration dependence of increases in %S phase (Fig. 4B) (41). The experiments whose results are presented in Fig. 4 thus demonstrate a close relationship between cyclin D1, D3, and E mRNA abundance and entry into S phase in T-47D breast cancer cells.

Progestin regulation of cyclin D1 gene expression. Previous experiments had demonstrated that synthetic progestins induce a transient increase in the rate of cell cycle progression of actively cycling T-47D cells, apparently by actions in early G_1 phase (39). Therefore, since the rapid increase in the abundance of cyclin D1 after insulin stimulation suggested that this cyclin might act during the early part of G_1 phase to determine rates of progression, changes in its expression after progestin treatment were examined. For these experiments T-47D cells cultured in serum-free medium supplemented with insulin were used. The initial synchrony of insulin-stimulated cells decays in subsequent cell cycles, and after several days' treatment, exponential growth with a stable cell cycle phase distribution is achieved (39, 41).

In T-47D cells cultured under these conditions, cyclin D1 was transiently induced by treatment with the synthetic progestin ORG 2058 (10 nM) (Fig. 5). The time course of cyclin D1 induction was delayed compared with that of c-*myc* (Fig. 5) (39). Maximum induction, which was two- to fourfold, was reached at 3 to 6 h, but increased expression of cyclin D1 was apparent within 2 h (Fig. 5), a time course of induction similar to that observed after insulin treatment. Progestin-stimulated cells begin to enter S phase after approximately 8 h of treatment (39) (Fig. 5), while insulin-

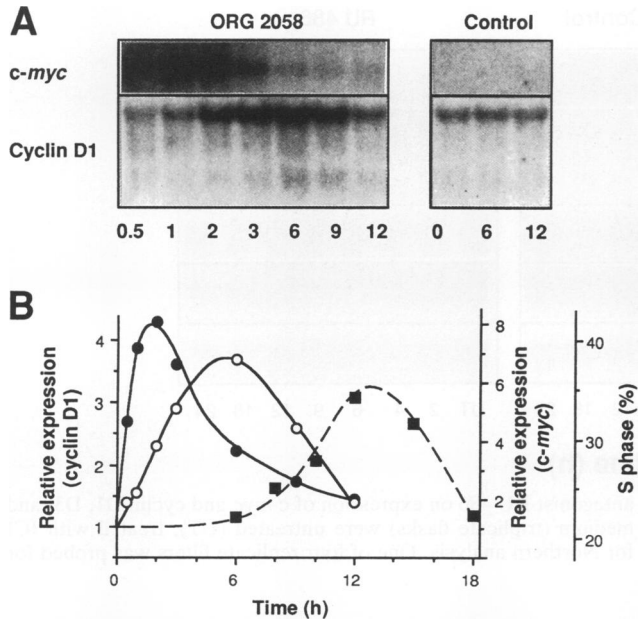

FIG. 5. Effect of the progestin ORG 2058 on the expression of c-*myc* and cyclin D1 mRNA. T-47D cells proliferating in insulin-supplemented serum-free medium (triplicate flasks) were treated with ORG 2058 (10 nM) and harvested at intervals for Northern analysis. A single filter containing 20 μg of total RNA per lane was sequentially probed for each mRNA species. Points in panel B were obtained by densitometric analysis of autoradiographs of c-*myc* (●) or cyclin D1 (○). The data are presented relative to the average for three vehicle-treated control samples harvested over the duration of the experiment. S-phase data (■) have been redrawn from reference 39.

stimulated cells enter S phase approximately 4 h later (Fig. 1). The earlier entry into S phase after progestin treatment was reflected in a shorter duration of elevated cyclin D1 gene expression. Cyclin D1 mRNA levels began to decrease between 6 and 9 h after progestin treatment and had returned to near control levels by 12 h, while in contrast, 12 h after insulin treatment cyclin D1 mRNA levels remained at the maximum. Thus, after either progestin or insulin treatment, cyclin D1 expression was high during G_1 phase and decreased as cells entered S phase.

Progestin effects on cell cycle progression can be antagonized by the addition of the progestin antagonist RU 486, added as late as 3 h after the beginning of ORG 2058 treatment; addition of the antiprogestin at later times only partially prevents the progestin stimulation of cell cycle progression (39). Therefore, if the induction of cyclin D1 is a progesterone receptor-mediated event involved in the acceleration of the cell cycle by progestins, it would be predicted to be inhibited by an antiprogestin. To test this prediction, T-47D cells were treated with progestin (ORG 2058, 10 nM), and antiprogestin (RU 486, 100 nM) was added either simultaneously or 3 h later. The cells were harvested 6 h after the beginning of progestin treatment, when cyclin D1 expression was clearly elevated and just prior to detectable changes in the %S phase. Figure 6 shows that treatment with RU 486 alone for 6 h had no significant effect on cyclin D1 gene expression but that simultaneous administration of ORG 2058 and RU 486 over the same period led to antagonism of the significant induction by ORG 2058. If RU 486 was added 3 h after the beginning of progestin treatment, when cyclin

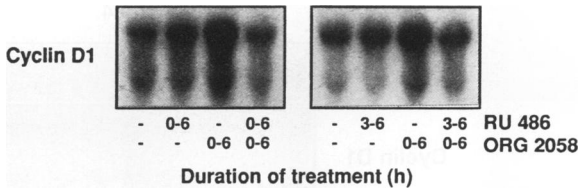

FIG. 6. Antagonism of progestin effect on cyclin D1 expression by antiprogestin. T-47D cells proliferating in insulin-supplemented serum-free medium were treated with progestin (ORG 2058, 10 nM) or vehicle and harvested 6 h later for Northern analysis. Parallel flasks were also treated with antiprogestin (RU 486, 100 nM) added either simultaneously or 3 h later than progestin or vehicle.

D1 mRNA levels had already doubled, cyclin D1 mRNA levels in treated cells returned to control levels by 6 h (Fig. 6 and data not shown). These data suggest that continued cyclin D1 gene expression beyond 3 h is necessary for cells to progress into S phase following progestin treatment and further support the close relationship between cyclin D1 expression and entry into S phase following mitogenic stimulation of breast cancer cells (Fig. 3 to 5).

Effect of growth arrest in G_1 phase on cyclin gene expression. The relationship between cyclin D1, D3, and E induction and stimulation of T-47D breast cancer cell cycle progression implies that inhibition of cell cycle progression would be accompanied by a decrease in the expression of one or more G_1 cyclin genes. Changes in cyclin gene expression associated with growth arrest in G_1 phase were measured in T-47D cells grown to high cell density in insulin-supplemented serum-free medium. The reduced growth rate as cells ceased exponential growth and entered plateau phase was accompanied by a decrease in histone H4 expression and by decreases in the expression of each of the cyclins D1, D3, and E (Fig. 7). Similarly, as expected from evidence for induction of cyclins D1, D3, and E after insulin treatment (Fig. 3), cells which were growth arrested in

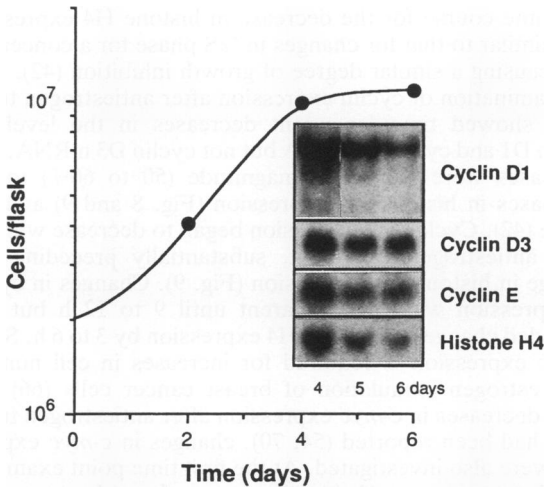

FIG. 7. Effect of growth arrest on the expression of cyclins D1, D3, and E. T-47D cells proliferating in insulin-supplemented serum-free medium were harvested for cell counts and/or Northern analysis during transition into plateau phase growth. RNA was harvested on days 4, 5, and 6 after the change to insulin-supplemented serum-free medium. One of two replicate filters has been probed for each gene.

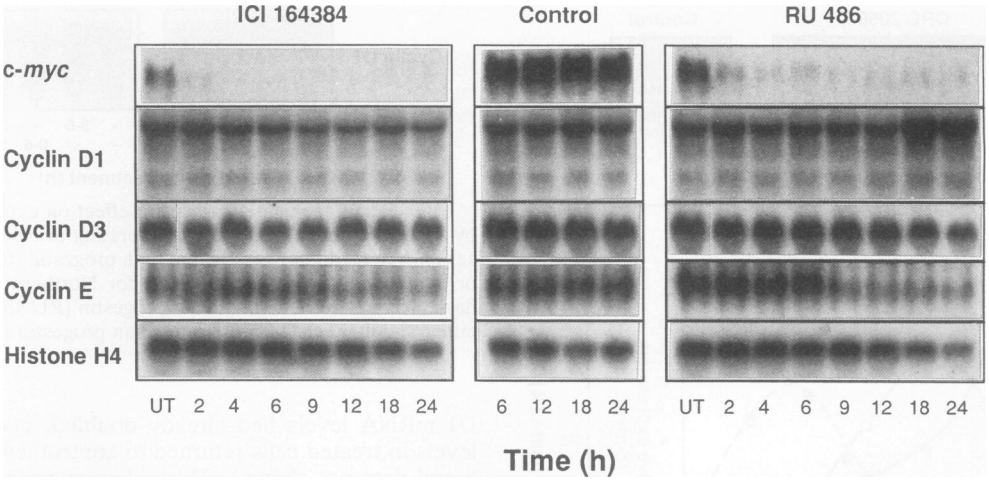

FIG. 8. Effect of the estrogen antagonist ICI 164384 and the progestin antagonist RU 486 on expression of c-*myc* and cyclin D1, D3, and E mRNA. T-47D cells proliferating in insulin-supplemented serum-free medium (triplicate flasks) were untreated (UT), treated with ICI 164384 (500 nM), RU 486 (100 nM), or vehicle and harvested at intervals for Northern analysis. One of four replicate filters was probed for each mRNA species.

serum-free medium expressed lower levels of these cyclins than cells proliferating exponentially after insulin stimulation in serum-free medium (data not shown).

Regulation of cyclin gene expression by steroid antagonists. Arrest of T-47D cells in G_1 phase can result not only from environmental signals, e.g., nutrient deprivation and cell-cell contact, but also from the effects of specific growth-inhibitory compounds like antiestrogens and antiprogestins. Changes in gene expression associated with these different arrest states were compared. The antiestrogen ICI 164384 inhibits the insulin-stimulated growth of T-47D cells in serum-free medium, in the apparent absence of any estrogen-induced mitogenic stimulus (42). As shown in Fig. 8 and 9, growth inhibition after ICI 164384 treatment (500 nM) was accompanied by a time-dependent decrease in histone H4 expression, beginning approximately 12 h after treatment. The time course for the decrease in histone H4 expression was similar to that for changes in %S phase for a concentration causing a similar degree of growth inhibition (42).

Examination of cyclin expression after antiestrogen treatment showed time-dependent decreases in the levels of cyclin D1 and cyclin E mRNA but not cyclin D3 mRNA. The decreases were similar in magnitude (50 to 60%) to the decreases in histone H4 expression (Fig. 8 and 9) and %S phase (42). Cyclin D1 expression began to decrease within 4 h of antiestrogen treatment, substantially preceding any change in histone H4 expression (Fig. 9). Changes in cyclin E expression were not apparent until 9 to 12 h but still preceded changes in histone H4 expression by 3 to 6 h. Since c-*myc* expression is required for increases in cell number after estrogen stimulation of breast cancer cells (66) and since decreases in c-*myc* expression after antiestrogen treatment had been reported (54, 70), changes in c-*myc* expression were also investigated. At the first time point examined in this experiment (2 h), the expression of c-*myc* was profoundly decreased, by approximately 95% (Fig. 8 and 9). In other experiments, small decreases in c-*myc* mRNA were consistently observed within 30 min of treatment (data not shown). These data are in accord with data obtained using other, structurally distinct antiestrogens (54, 70). The changes in gene expression associated with growth arrest after antiestrogen treatment were thus compatible with a role for c-*myc* and cyclin D1, and, to a lesser extent, cyclin E, in the control of breast cancer cell cycle progression by antiestrogens.

The proliferation of T-47D cells in insulin-supplemented serum-free medium can also be inhibited by antiprogestin treatment, in the absence of progestin (42). Changes in cell cycle phase distribution occur over similar time frames after

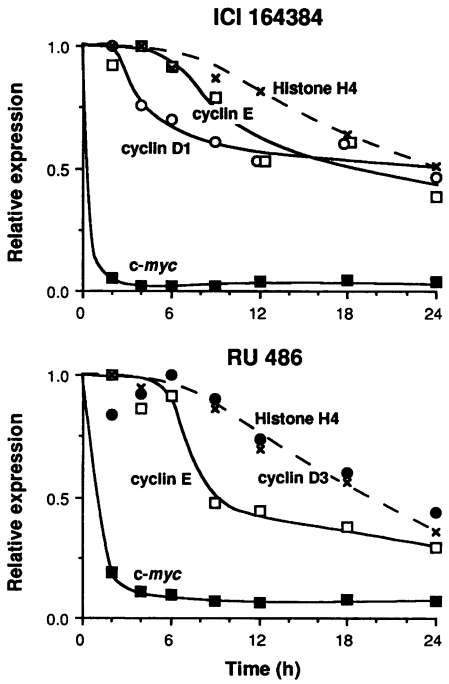

FIG. 9. Effect of the estrogen antagonist ICI 164384 and the progestin antagonist RU 486 on the relative expression of c-*myc* and cyclins D1, D3, and E. Densitometric analysis of data presented in Fig. 8 is presented relative to the maximum expression observed during the experiment.

antiestrogen and antiprogestin treatments (42), and therefore the changes in c-*myc* and cyclin gene expression after treatment with antiprogestin (RU 486, 100 nM) and antiestrogen were compared. Growth inhibition after antiprogestin treatment was accompanied by an approximately 50 to 60% decrease in levels of histone H4 mRNA which began between 12 and 18 h (Fig. 8 and 9). Like antiestrogen treatment, antiprogestin treatment decreased c-*myc* expression by more than 80% within 2 h. While cyclin D1 expression decreased after antiestrogen treatment, the antiprogestin failed to affect the expression of this gene (Fig. 8). Small increases, typically of 20 to 30%, were observed in some experiments (for an example, see Fig. 8) but were inconsistent and displayed no clear time dependence. In contrast with cyclin D1 expression, cyclin D3 expression, which was unaffected by antiestrogen treatment, was markedly decreased by antiprogestin treatment, to <50% of control values (Fig. 8 and 9). This decrease began after 12 h of treatment and was apparently coincident with the decrease in %S phase. Changes in the level of cyclin E mRNA were not apparent until approximately 9 h after RU 486 treatment, preceding changes in histone H4 expression by 3 h or more (Fig. 8 and 9). Thus, although antiestrogens and antiprogestins have similar effects on T-47D cell cycle progression and c-*myc* gene expression, clear differences in the regulation of G_1 cyclin genes were observed.

DISCUSSION

Cyclin gene expression and cell cycle progression. Although it has been hypothesized that regulation of G_1 cyclin gene expression is central to passage through G_1 phase in mammalian cells (17, 53), this regulation has not yet been examined with many different cell types. Our data demonstrate sequential induction of cyclin gene expression with progress through the cell cycle following stimulation of growth-arrested T-47D cells with insulin. The responses of cyclins A and B1 and of *cdc2* and *cdk2* in these breast carcinoma cells are consistent with results from other cell types, in which cyclin A, *cdc2*, and *cdk2* mRNA expression increases in late G_1 phase (10, 24, 33, 44, 50) and cyclin B1 expression increases during G_2 phase (49). The level of *cdc2* expression in T-47D cells arrested in serum-free medium is much greater than the barely detectable levels observed in quiescent T lymphocytes or fibroblasts (13, 24). Furthermore, the modest degree of induction is consistent with differences between *cdc2* mRNA levels in synchronized HeLa cells during G_1 and S phase (33). These data support our conclusion that T-47D cells in serum-free medium are arrested early in G_1 phase rather than in a G_0 quiescent state (41).

In T-47D cells, increased expression of cyclin E was apparently coincident with entry into S phase and declined as cells passed through S phase. A similar pattern of regulation has been observed in other cultured cells (27, 56), consistent with evidence that cyclin E protein and the associated kinase activity reach their maximum in late G_1-early S phase (9, 21). These data indicate that this cyclin might regulate the G_1-to-S-phase transition rather than earlier events in the mammalian cell cycle. Regulation of cyclin C with cell cycle progression after insulin stimulation was not detected, arguing against transcriptional control of this gene in the regulation of T-47D cell cycle progression. However, since the magnitude of changes in the expression of cyclin C is in general smaller than that of the changes in the expression of other cyclins (27, 56), it is possible that the proportion of cells entering S phase in the experiments reported here was not sufficient for changes in cyclin C expression to be detected. Alternatively, serum deprivation may arrest T-47D cells at a point in the cell cycle after cyclin C induction, although evidence that cyclin C expression increases with progress through G_1 phase to reach a maximum in early S phase in a growth factor-stimulated murine pre-B-cell line argues against this conclusion (56).

The greatest diversity in expression and regulation among cyclin genes occurs in members of the cyclin D family. Some cell types express all three D-type cyclins, but many express only two, suggesting that these genes may perform different roles in different cell types (18, 32, 68, 69). In T-47D cells, cyclin D1 expression was greatest during G_1 phase and decreased as cells entered S phase. A similar pattern of cyclin D1 expression after mitogenic stimulation of several cell types, including normal human mammary epithelial cells, has been reported (32, 36, 69). Although differences in the precise cell cycle phase dependence of cyclin D1 regulation have been observed, a high level of expression during G_1 is common to all the published studies (30, 32, 36, 69). Cyclin D3 regulation paralleled cyclin D1 expression in serum-stimulated fibroblasts, peaking in late G_1 phase (69), but was an early response to cytokine stimulation of bone marrow-derived pre-B-cell lines (56). In both T-47D cells and normal mammary epithelial cells, cyclin D3 gene expression increased in mid-G_1 phase and remained elevated as the cells progressed into S phase (36; also this study). Thus, cyclin D3 regulation more closely mimicked regulation of cyclin E than cyclin D1 in breast epithelium, arguing against coordinate regulation of cyclins D1 and D3, which had been suggested on the basis of their similar kinetics of induction in fibroblasts (69).

Induction of cyclins D1, D3, and E in T-47D cells was not restricted to insulin stimulation but was also observed after treatment with other potent breast cancer mitogens, i.e., IGF-I and FCS (Fig. 4) and basic fibroblast growth factor (unpublished data). Thus, increased cyclin D1 mRNA expression is a common response to mitogenic stimulation by peptide growth factors in these cells. The rapid response of cyclin D1 suggests that it may be intimately associated with the initiation of progress through G_1 phase. Furthermore, the proportion of cells which later entered S phase appeared to be related to the degree of induction of cyclins D1, D3, and E. A similar conclusion was reached from studies using murine macrophages, in which the proportion of cells which entered S phase was related to the level of cyclin D1 protein present late in G_1 phase (32). This relationship between cyclin mRNA abundance and entry into S phase suggests that induction of expression of cyclins D1, D3, and E is involved in the commitment to DNA synthesis in breast cancer cells. Furthermore, the similarity between the patterns of cyclin gene expression in mitogen-stimulated normal mammary epithelial cells (36) and T-47D breast cancer cells suggests that these cyclins play a similar role in the regulation of proliferation in both normal and neoplastic mammary epithelium.

Progestin regulation of cell cycle progression. Progestin treatment of T-47D cells in insulin-supplemented serum-free medium stimulates a cohort of cells to enter S phase after approximately 8 h (39). The rate of entry into S phase is approximately twice that of control cells, resulting from an increase in the rate of cell cycle progression of actively cycling cells rather than from entry of quiescent cells into the cycle (39). Acceleration of cells already in cycle can be achieved only by action on processes governing progression

through G_1, and thus, progestins appear to stimulate a rate-limiting step. Evidence that the yeast G_1 cyclins are rate limiting (references 48 and 53 and references therein) raises the possibility that mammalian G_1 cyclins are also rate limiting, although this has yet to be demonstrated. If this hypothesis is correct, then progestin induction of a G_1 cyclin could account for the acceleration of progress through G_1 phase observed after progestin treatment. The rate-limiting process accelerated by progestins is completed 5 h or more before entry into S phase (39) and therefore occurs in the first half of G_1 phase. The rapid induction of cyclin D1 after insulin stimulation of progress through G_1 phase suggested an action at a time consistent with these cell cycle kinetic effects of progestins. This study demonstrates increased cyclin D1 gene expression within 2 h of progestin treatment which was sustained as the progestin-stimulated cells moved through G_1 phase.

The antiprogestin RU 486 reverses progestin effects on proliferation in a time-dependent manner. Addition of antiprogestin 3 h or less after progestin treatment almost completely inhibits the effect of the progestin, but the effect of the antiprogestin is decreased with increasing delay in the time of administration, allowing progressively more cells to be accelerated through G_1 phase by the progestin (39). Antiprogestin addition to progestin-treated cells (whether simultaneous with progestin addition or after a 3-h delay) returned cyclin D1 mRNA levels to near-control values at 6 h. This occurred even though cyclin D1 mRNA levels were approaching their maximum 3 h after progestin treatment. Thus, antiprogestin treatment substantially reduced the duration of cyclin D1 elevation. Delaying the addition of antiprogestin further, until the cyclin D1 mRNA levels decline (>6 h), would be expected to have relatively minor consequences for the cyclin D1 mRNA levels, consistent with the failure of antiprogestin addition at this time to prevent progestin stimulation of proliferation (39). The similar consequences of antiprogestin addition for the duration of elevated cyclin D1 mRNA levels (this study) and on cell cycle progression (39) suggest that these two processes are linked. Progestin induction of c-*myc* expression is also reversible by simultaneous antiprogestin treatment (39). However, induction of c-*myc* expression is both rapid and transient, and by 3 h, c-*myc* mRNA levels are beginning to decline. It might be expected, therefore, that addition of antiprogestin 3 h after progestin treatment would be too late to prevent the consequences of c-*myc* induction. Thus, regulation of cyclin D1 appears to more closely correlate with progestin regulation of proliferation than does regulation of c-*myc*.

In murine macrophages, colony-stimulating factor 1 stimulation increases the level of cyclin D1 gene expression (32). If colony-stimulating factor 1 is withdrawn, both cyclin D1 mRNA and protein rapidly degrade with half-lives of <2.5 h (32). This situation is analogous to that in cells treated with progestin and subsequently treated with antiprogestin: antiprogestin prevents continued induction of cyclin D1 expression by progestins, and cyclin D1 mRNA levels decline rapidly. Within 3 h of antiprogestin addition to progestin-treated cells, the cyclin D1 mRNA declined from an induced level almost to the control level, suggesting that the cyclin D1 mRNA half-life in T-47D cells under these experimental conditions is similar to that observed in murine macrophages.

Steroid antagonist effects. The induction of cyclin D1 gene expression within 2 h of mitogenic stimulation, described above, is compatible with a role for this gene in early G_1 phase, when breast cancer cells are sensitive to the inhibitory effects of antiestrogens and antiprogestins (42, 43, 60). Therefore, growth inhibition by antiestrogens might be predicted to be associated with a decrease in the expression of cyclin D1. In agreement with this proposition, treatment with the antiestrogen ICI 164384, a potent inhibitor of breast cancer cell cycle progression (43, 64, 65), reduced the expression of cyclins D1 and E to a degree similar to the reduction in %S phase or histone H4 expression. The decrease in cyclin D1 and E expression clearly preceded changes in cell cycle phase distribution. Thus, the regulation of cyclin D1 and E expression by ICI 164384 is not merely a consequence of growth arrest. Again, the change in cyclin D1 gene expression, apparent at 4 h, was an early response to regulation of proliferation, strengthening the conclusion that cyclin D1 regulation is central to the regulation of cell cycle progression in T-47D breast cancer cells.

The similar kinetics of the changes in cell cycle phase distribution after antiestrogen or antiprogestin treatment argued that these compounds might inhibit cell cycle progression by convergent pathways (42). Both compounds decreased cyclin E expression, and although the magnitude of the decline was similar to the magnitude of the subsequent decline in %S phase, these changes were relatively late responses to antagonist treatment. However, while antiestrogen treatment rapidly decreased cyclin D1 expression but had no effect on cyclin D3 expression, antiprogestin treatment did not affect cyclin D1 expression but decreased cyclin D3 expression. The reduced expression of cyclins D3 and E after antiprogestin treatment did not occur until 9 h or more after the commencement of treatment, and therefore, although they may still be part of the mechanism by which entry into S phase is inhibited in the presence of antiprogestins, it is unlikely that the decreases in expression of cyclins D3 and E are primary responses. Whether the decreased expression of one or a combination of these cyclins is sufficient to cause growth arrest is unknown but is currently under investigation. However, inhibition of c-*myc* expression with antisense oligonucleotides has been shown to prevent entry into DNA synthesis in a number of cell types (13, 15, 16). Thus, the >90% decrease in c-*myc* expression within 2 to 4 h after antiestrogen or antiprogestin treatment could be sufficient for growth arrest.

That the expression of some cyclins was maintained despite growth arrest after antiestrogen or antiprogestin treatment was an unexpected result. Growth arrest in T-47D cells after serum deprivation or growth to plateau phase was associated with decreased expression of cyclins D1, D3, and E; similar results have been obtained with other cell lines (21, 69). Furthermore, a number of antiproliferative agents inhibit cyclin D1 expression in bone marrow-derived macrophages (7). After stimulation of proliferation by either insulin or progestin, the expression of cyclin D1 remained high as the stimulated cells moved through G_1 phase but decreased as the cells entered S phase. Similarly, in murine macrophages, synthesis of cyclin D1 protein declined during S phase, although the level of the mRNA remained high (32). This suggests the existence of regulatory mechanisms, normally triggered near the G_1-S boundary, which limit the production of cyclin D1 mRNA and/or protein in S-phase cells. The maintenance of cyclin D1 mRNA levels in RU 486-treated cells may reflect a failure to complete a step which is prerequisite for this regulatory mechanism. It is important to note, however, that despite evidence that cyclin B destruction is necessary for exit from mitosis (38), there is no evidence that cyclin degradation is necessary for exit

from G_1 phase (17), and thus, an elevated cyclin D1 level would not be expected to be a cause of growth arrest.

Conclusions. The data presented in this article show that regulation of G_1 cyclin gene expression is closely associated with changes in the proliferation rate of T-47D breast cancer cells, not only following stimulation with peptide and steroid mitogens but also upon growth arrest by steroid antagonists. The similarities between cyclin D1 and D3 regulation in growth factor-stimulated normal breast cell lines and in neoplastic breast cell lines (36; also this study) argue that steroidal regulation of these genes may also occur in normal breast epithelial cells. A key role in the control of breast epithelial cell cycle progression implicates altered expression of these G_1 cyclins in aberrations in the growth control of some breast tumors (17). Indeed, cyclin D1 is amplified in approximately 15% (2, 61), and overexpressed in up to 45% (5), of breast tumors. In addition, the difference in cyclin gene regulation by antiestrogens and antiprogestins suggests a mechanism by which tumors which are relatively insensitive to one compound may retain sensitivity to other hormonal therapies.

Induction of c-*myc* expression appears to be necessary for estrogen-induced cell cycle progression (66). However, the critical event for progestin stimulation of cell cycle progression appears to be cyclin D1 induction rather than c-*myc* induction. Whether induction of either gene alone is sufficient for eventual entry into DNA synthesis has not been demonstrated in breast cancer cells, but evidence from other cell types suggests that the products of multiple genes are required to trigger the events leading to DNA synthesis (3). The data presented in this article are consistent with the interpretation that stimulation of breast cancer cell cycle progression requires the induction of both c-*myc* and cyclin D1. However, decreased expression of either gene may be sufficient for growth inhibition. Whether the regulation is mediated directly or indirectly by steroid hormones and their antagonists, the data support the hypothesis that regulation of the expression of c-*myc* and G_1 cyclins could prove to be the basis for steroidal regulation of proliferation.

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