Regulation of Cyclin B1 by Estradiol and Polyamines in MCF-7 Breast Cancer Cells

Thresia Thomas and T. J. Thomas

Department of Environmental and Community Medicine, Environmental and Occupational Health Sciences Institute [T. J.T.], University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

ABSTRACT

Recent studies have identified a family of proteins called cyclins that control cell cycle. Among these proteins, cyclin B synthesis and degradation are necessary and sufficient to cause a Xenopus egg cell-free system to oscillate between S and M. To understand the link between hormonal regulation of cell growth and the expression of B-type cyclins, we studied the effect of estradiol on cyclin B1 mRNA in a hormone-responsive breast cancer cell line, MCF-7. Cells were synchronized at G1 by isoleucine starvation, and estradiol was added along with the removal of cell cycle block. Flow cytometric analysis showed 81 ± 7% cells in G1 after 30 h of isoleucine starvation. Significant population of cells progressed to S by 16 h after the addition of estradiol, whereas a comparable transition occurred in control cells by 36 h only. In cells progressing from G1 to G2/M under the influence of estradiol, there was a significant increase in cyclin B1 mRNA at 30 and 36 h, consistent with the accumulation of this cyclin in G2/M. In addition, we found that cyclin B1 mRNA degradation occurred early in G2, and this process was accelerated by estradiol. At 2 h after removal of the isoleucine block, there was a 40% reduction in the level of cyclin B1 mRNA in estradiol-treated cells compared to untreated controls. Cyclin B1 protein degradation followed a similar pattern, as determined by Western blots using a monoclonal anti-cyclin B1 antibody.

INTRODUCTION

Estradiol exerts pleiotropic growth stimulatory effects on breast epithelial cells and other sex accessory tissues as part of their normal development (1–3). Estradiol is also involved in the induction and progression of breast cancer, and a subset of breast cancers regresses by ablation of estradiol. Estrogen receptor, a protein that mediates the action of estradiol, is present in the majority of breast tumors, and its presence correlates with responsiveness to the tumor to antiestrogen therapy (4–6). Several pathways have been proposed for estrogenic regulation of breast cancer cells (7–10): (a) direct gene regulatory effects mediated by estrogen receptor facilitate the expression of estrogen receptor-dependent genes such as c-fos, c-myc, and ODC; (b) estradiol exerts its effects on cell growth by stimulating the production of growth factors, particularly epidermal growth factor and insulin-like growth factor I (8, 9); (c) estradiol may release the growth-inhibitory effects of certain serum-derived factors and thereby enhance cell growth (10). Despite extensive work on the involvement of estradiol in breast cancer, the control of the cell cycle and cell proliferation by this hormone is not fully understood.

Several of the molecules that control cell cycle have been recently identified (11–13). Two major regulatory points in eukaryotic cell cycle are at G1 and G2/M transitions. In the G2/M transition, the protein complex, MPF, drives interphase cells into mitosis (14–16). A component of MPF, cyclin was originally named as a result of its unusual pattern of protein expression during early embryonic cycles of sea urchin eggs and clams. The protein kinase activity of MPF is activated only after its association with cyclins. Pines and Hunter (17, 18) characterized human cyclins A and B1 as the mitotic cyclins because these proteins induced maximal activation of associated protein kinase in G2/M. Another group of cyclins including C1, D1, D2, D3, and E were described later as G1 cyclins (19, 20). In spite of this classification, mutant yeast that were deficient in G1 cyclins could be rescued by mitotic cyclins, suggesting that the functional domains of these proteins might be acting in a similar manner. Recent studies also suggest that cyclin B protein has multiple functions including the activation of a specific tyrosine phosphatase (21). Thus regulation of G1, as well as mitotic cyclins might be important in the control of breast cancer cell growth. In this report, we describe the effect of estradiol on the control of cyclin B1 mRNA and protein in synchronized MCF-7 cells.

Early studies on the action of estrogens in target tissues showed that this hormone stimulates ODC activity (22). ODC is a key enzyme in the biosynthesis of the naturally occurring polyamines, putrescine [H2N(CH2)2NH2], spermidine [H2N(CH2)3NH(CH2)2NH2], and spermine [H2N(CH2)3NH(CH2)2NH(CH2)2NH2]; Refs. 22–24. Recent studies suggest that the polyamine pathway is interlinked with estrogenic regulation of cell growth in breast cancer cells (25–28). The inhibitory effect of antiestrogens on breast cancer cells could be reversed by the addition of polyamines (25, 27). Furthermore, polyamines are capable of altering the structural organization and DNA binding of estrogen and other steroid receptors (28–30). Therefore, we questioned whether estrogenic control of cell cycle and the expression of cyclin B1 mRNA were related to the accumulation of polyamines in these cells. For these studies, we used an inhibitor of ODC, DFMO, for polyamine depletion and exogenous putrescine, spermidine, or spermine homologues for polyamine repletion.

Our results indicate that estradiol accelerates the down-regulation of cyclin B1 mRNA in early G1, and its accumulation in S and G2/M. Our data further indicate an important role for polyamines in the rapid degradation of cyclin B1 mRNA in early G1.

MATERIALS AND METHODS

Chemicals and Reagents. DFMO was a gift from Peter P. McCann of the Marion Merrell Dow Research Institute, Cincinnati, OH. C4, spermidine-3HCl, spermine-4HCl, and aminoguanidine were obtained from Sigma Chemical Co. (St. Louis, MO). Putrescine analogues, C3, C5, and C6, (putrescine); C5, 1,5-diaminopentane; C6, 1,6-diaminohexane; DCC, dextran-coated charcoal; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.
were obtained in the form of their dihydrochlorides from Aldrich Chemical Co. (Milwaukee, WI). Cyclin B1 cDNA probe was a gift from Drs. Pines and Hunter of the Salk Institute, La Jolla, CA. Monoclonal anti-cyclin B1 antibody was obtained from Pharmingen (San Diego, CA). Chemiluminescence based a Western blot detection system was obtained from Amsersham (Arlington Heights, IL).

Cell Culture. MCF-7 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, 2 μg/ml insulin, and 10% fetal calf serum (Hyclone, Salt Lake City, UT; Ref. 27). Culture medium, trypsin, antibiotics, and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY. One week before each experiment, MCF-7 cells were grown in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum which was pretreated with DCC. For this purpose, we used a stock DCC suspension consisting of 0.5% Norit A, 0.05% Dextran T-70, 10 μM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.25 mM sucrose. This suspension (200 ml) was centrifuged at 5000 × g for 10 min and decanted. Then, 200 ml of serum was added to the pellet and stirred at 4°C for 30 min. The mixture was centrifuged at 5000 × g for 10 min. The serum was decanted and sterilized by filtration through a 0.22 μm membrane. We conducted all experiments with MCF-7 cells in phenol red-free medium and DCC-treated serum to avoid estrogenic effect of phenol red (31).

In experiments with DFO, a 200-μM stock solution of this compound was made in double distilled water, pH adjusted to 7.4, and small volumes were added to the culture medium. In polyamine repletion experiments, cells were treated with polyamines along with 1 μM aminoguanidine in order to inhibit serum-derived amine oxidase from degrading exogenous polyamines.

Flow Cytometry. Cells were grown in 100-mm dishes, washed with PBS, and covered with a buffer containing 40 μM sodium citrate, 250 mM sucrose, and 5% dimethylsulfoxide; then cells were frozen at −70°C (32, 33). On the day of RNA extraction, cells were thawed, and the centrifuge buffer was removed. Cells were then trypsinized for 10 min and further treated with a solution containing trypsin inhibitor and RNase for 10 min. Cells were then stained by adding propidium iodide solution in sodium citrate buffer and analyzed by a Coulter flow cytomter.

RNA Extraction. RNA was extracted using the guanidium isothiocyanate procedure (34). Cells (2 × 106) were seeded in 100-mm culture dishes. After 48 h, medium was removed, and cells were treated for 30 h with isoleucine-free medium. Estradiol or ethanol vehicle was then added for specific time periods. Estradiol was dissolved in ethanol as a concentrated stock solution to maintain ethanol concentrations below 0.1%. The cells were trypsinized (0.25% trypsin in 2.5 mM EDTA), washed once in cold PBS (pH 7.2), and centrifuged at 800 × g for 5 min. The pellet was resuspended in 3 ml of a solution containing 5 mM guanidium isothiocyanate, 50 mM Tris-HCl (pH 7.6), and 5% β-mercaptoethanol. This cell lysate was immediately layered on a 5.7-M CsCl cushion and centrifuged at 114,000 × g for 18 h at 20°C. The RNA pellet was resuspended in 1 ml of a buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% SDS. The RNA solution was extracted with a 4:1 mixture of chloroform and butanol, precipitated with absolute ethanol, and resuspended in sterile water. The RNA concentration was determined by measuring the optical density at 260 nm.

Northern Blot Hybridization. RNA prepared from MCF-7 cells was separated by agarose (1%) gel electrophoresis under denaturing conditions (35). Ethidium bromide (50 μg/ml) was added to samples immediately before loading. The gel and running buffer contained 2.2 M formaldehyde, 50 mM morpholino-propanesulfonic acid, 10 mM sodium acetate, and 1 mM EDTA (pH 7.0. 18S and 28S RNA from calf thymus (P. L. Biochemicals, Piscataway, NJ) was used as molecular size markers. The integrity of the 18S and 28S bands of the extracted RNA indicated that RNA molecules have not degraded during the extraction process. RNA was transferred onto a nylon membrane by a modified version of the Maniatis procedure (35) for the ProbeTech 2 automated transfer system (Oncor, Gaithersburg, MD). Slot blots were prepared by applying purified RNA at 0.5 and 1 μg levels on nylon membrane using a slot blot apparatus obtained from Bethesda Research Laboratories, Bethesda, MD.

The plasmid (pGEM 4Z) harboring human cyclin B1 cDNA sequences was cleaved by BamHI, and the insert was purified by a Gene Clean kit from Bio 101, La Jolla, CA. The DNA was recovered by ethanol precipitation and resuspended at a concentration of approximately 0.1 μg/ml in a buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 20 mM NaCl. The DNA was labeled with 32P using a nick translation reagent kit from Oncor. The RNA blots were hybridized with 32P-labeled cyclin B1 cDNA. Hybridization conditions and washing procedures were optimized using standard protocols (36). The specific activity of the cDNA probe was in the range of 5–10 × 107 cpm/μg DNA. Prehybridization was for 1 h using Hybrisol I (Oncor) containing 50% formamide and other salts adapted from Maniatis et al. (37). Hybridization was conducted in the same buffer at 45°C for 20 h. Filters were washed twice with buffer containing 0.1X sodium saline citrate and 0.1% SDS at 22°C for 15 min. Subsequently, filters were washed for 1 h at 52°C in the same washing buffer. Washed filters were blotted on a paper towel and then exposed to Kodak X-Omat AR film for 24 to 72 h at −70°C before development. The radioactive cyclin B1 probe was removed by boiling the filter in RNase-free water, and the filter was rehybridized using 32P-labeled β-actin probe (Oncor).

Western Blots for Protein B1 Protein. Experiments on the effects of estradiol on cyclin B1 protein accumulation/degradation were conducted as described above for RNA preparation. Cell pellets were lysed in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 0.5% SDS and 1 mM dithiothreitol. Samples were boiled for 5 min and then diluted with 400 μl RIPA buffer (10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM (ethylenedinitritro)-tetra acetic acid, 50 mM sodium fluoride, 100 μM sodium vanadate; and 1% aprotinin; Ref 37). Samples were centrifuged at 29,000 × g for 1 h, and the supernatant was used for the analysis. Proteins (20 μg) from the cellular extract were separated by 10% polyacrylamide gel. Electrophoresis was performed using a Bio-Rad minigel system. The proteins were transferred to PVDF immobilon membrane (Bedford, MA) and probed with a purified monoclonal mouse anti-human cyclin B1 protein antibody at 1:500 dilution. Cyclin proteins were then visualized using horseradish peroxidase-labeled anti-mouse secondary antibody (1:5000 dilution) with a chemiluminescence-based detection system.

Measurement of Intracellular Polymamines. Approximately 3 × 105 cells were harvested from culture, washed with PBS, and pelletized. The cell pellet was treated with 300 μl of 8% sulfosalicylic acid and sonicated for 15 s in ice. The solution was incubated on ice for 1 h and centrifuged at 900 × g for 5 min to remove the precipitated protein. Intracellular polynamine levels were determined by HPLC after derivatization to their dansyl derivatives as described by Singh et al. (38). C6 was used as an internal standard. HPLC was performed on a Perkin-Elmer system using a Binary LC Pump 250 and a Fluorescence Detector LS 40.

Statistical Analysis. Statistical significance of difference between control and treatment groups was determined by the Student’s t test using the computer program Statview.

RESULTS

Synchronized MCF-7 cells were seeded in phenol red-free medium containing charcoal-treated serum for 1 week prior to the experiment. Growth of MCF-7 cells under these conditions was necessary to obtain maximal sensitivity to estradiol and to avoid the estrogenic effects of phenol red (27, 31). Two days after the experimental seeding, cells were fed with isoleucine-free medium for 30 h in order to arrest cells in G1. Estradiol was added along with the removal of the cell cycle block with isoleucine containing medium. Table 1 shows the results of cell cycle analysis by flow cytometry as a percentage of cells in different phases of cell cycle. Eighty one % of the cells were in G1 after isoleucine starvation. There was no significant change in the distribution of cells 12 h after the release of cell cycle block. Marked changes between the percentages of G1 and S cells were observed at 16 h after the addition of estradiol. Significant difference (P < 0.05; n = 4) from control and estradiol-treated cultures was also observed at 16 h after treatment. In contrast, a marked change from G1 to S occurred to appear in control cells after 36 h.

In order to document the general pattern of cyclin B1 mRNA accumulation in estradiol-treated MCF-7 cells traversing through G1→S→M, we extracted total RNA at 0, 12, 16, 24, 30, and 36 h after estradiol addition using the guanidium isothiocyanate procedure (34).
RNA was analyzed by Northern blotting and hybridization was conducted with a 32P-labeled cyclin B1 cDNA probe. Fig. 1 shows changes in cyclin B1 mRNA levels during cell cycle progression from G1 to M. Results shown are from two experiments, one for 12 to 24 h and the other for 30 and 36 h time points. A major species of mRNA was observed at about 1.7 kilobases, but a second, larger mRNA was found in overexposed autoradiograms. Densitometer scanning of hybridization signal (after correction for variations in RNA levels) showed that estradiol induced a 10 to 20% increase in cyclin B1 mRNA levels at 12 to 24 h time points and a significant 37 and 56% increase (P < 0.05; n = 4, including slot blots) at 30 and 36 h time points. The latter time points mark the transition of MCF-7 cells to G2/M, although cells tend to lose synchrony by this time. Thus, by 36 h, an increase in the percentage of G1 cells is observed as cells reenter G1 after the first cell division in the presence of estradiol. These results would suggest that estradiol-induced facilitation of cell cycle involves an increase in cyclin B1 mRNA at G2/M.

Previous studies (17) on HeLa cells have shown that cyclin B1 mRNA degradation occurred in early G1. In order to examine whether this degradation is affected by estradiol, we examined the effects of estradiol on cyclin B1 mRNA in MCF-7 cells in early G1. For this purpose, total RNA was extracted from synchronized cells treated with estradiol at 2, 4, and 8 h after its addition. Control cells that were not exposed to estradiol were also harvested at these time points. Fig. 2A represents an autoradiogram from Northern blot analysis of cyclin B1 mRNA. There was a decrease in the intensity of cyclin B1 mRNA signal in estradiol-treated cells compared to controls. Fig. 2B shows the quantification of this decrease by a scanning densitometer. There was a significant 40% (P < 0.05; n = 3) decrease in cyclin B1 mRNA intensity in estradiol-treated cells compared to controls at 2 h after the removal of the cell cycle block. The decrease in cyclin B1 mRNA intensity continued for up to 8 h after the removal of the G1 block, and estradiol accelerated this process.

In the next set of experiments, we examined whether estradiol-induced alterations in cyclin B1 mRNA could be detected at the protein level. Cells harvested as in previous experiments were used for the analysis of cyclin B1 protein by Western blots using a monoclonal anti-cyclin B1 antibody. As shown in Fig. 3, two proteins were recognized by this cyclin B1 antibody; the upper band coincided with the reported molecular weight of 62,000 of cyclin B1 protein. The lower band appears to be a degradation product of the Mr 62,000 protein because we observed an increase in the intensity of this band at the 6-h time point as the intensity of the upper band decreased. The observation of immunoreactive cyclin B1 protein in these experiments is consistent with our finding of relatively high levels of the mRNA in G1. Estradiol accelerated the degradation of this protein as in the case of the mRNA. Quantification of the cyclin B1 protein (Fig. 3) showed that its degradation continued up to 12 h after the addition of estradiol, reaching 31 ± 8% of the control at 2 h. This decrease was statistically significant (P < 0.02; n = 3). Additional three experiments using immunoprecipitated cyclin B1 protein provided a similar pattern of cyclin B1 degradation (results not shown).

Table 1 Distribution of MCF-7 cells in cell cycle phases as measured by flow cytometry

<table>
<thead>
<tr>
<th>Time after the removal of G1 block (h)</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>81 ± 6</td>
<td>81 ± 6</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>12</td>
<td>87 ± 5</td>
<td>73 ± 4</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>16</td>
<td>83 ± 4</td>
<td>63 ± 6*</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>24</td>
<td>80 ± 2</td>
<td>31 ± 5*</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>36</td>
<td>74 ± 3</td>
<td>54 ± 5*</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

* Significantly different from controls (P < 0.05).

Fig. 1. Accumulation of cyclin B1 mRNA in cells progressing from G1. Cyclin B1 mRNA was detected in cells treated with estradiol for 0 (Lane 1), 12 (Lane 2), 16 (Lane 3), and 24 (Lane 4) h after treatment with estradiol by Northern blot analysis. Lane 5, RNA from cells cycling in the absence of estradiol for 24 h. Lane 6–10, results of a separate experiment. Lanes 6, 7, and 9, controls (no estradiol) at 0, 30, and 36 h; Lanes 8 and 10, estradiol-treated cells at 30 and 36 h. Arrow, cyclin B1 mRNA (1.7 kilobase pairs).

Fig. 2A, estradiol-induced down-regulation of cyclin B1 mRNA in early G1 by Northern blot analysis. Lanes 1, 3, and 5, controls (no estradiol) at 2, 4, and 8 h, respectively; Lanes 2, 4, and 6, estradiol-treated cells at 2, 4, and 8 h. B, quantitation of cyclin B1 mRNA signals using Hoefer GS 300 scanning densitometer from three Northern blot experiments representing control (C) and estradiol-treated (E) cells. Hybridization signals were normalized to the amount of RNA using negatives of gel pictures after ethidium bromide staining. Data are the mean ± SD of three separate experiments. Signals from estradiol-treated cells were significantly different from those of control cells at all time points tested (P < 0.05; n = 3).
REGULATION OF CYCLIN B1 BY ESTRA DiOL AND POLYAMINES

Fig. 3. Estradiol-induced down-regulation of cyclin B1 protein in G1. Proteins were detected by Western blots using a chemiluminescence detection system. Lanes 1, 3, and 5, controls; Lanes 2, 4, and 6, estradiol-treated samples at 2, 6, and 12 h, respectively, after the addition of estradiol and removal of the cell cycle block. Columns, the intensity of the protein bands quantitated by densitometer for control (○) and estradiol-treated (■) samples. Bars, ± SD in 3 separate experiments.

Fig. 4. Changes in cyclin B1 and β-actin mRNA intensity as a function of cell cycle progression. Membranes used for probing with cyclin B1 cDNA (○) were boiled to remove the radioactive probe and then hybridized with a β-actin probe (■). Autoradiograms from a 24-h exposure were used for densitometric scanning. Points, mean of four separate experiments, two Northern blots, and two slot blots.

We also analyzed the accumulation of cyclin B1 protein at 30 and 36 h time points which mark G2/M (results not shown). There was a 4-fold increase in the intensity of the protein band at 36 h compared to that at 12 h. However, the increase in intensity of the protein band at these time points in estradiol-treated cells was not statistically significant when compared to that of control cells at the same time points.

To compare the oscillation of cyclin B1 mRNA to the expression of a structural gene, each blot was boiled to remove cyclin B1 cDNA and then reprobed with 32P-labeled β-actin probe. Densitometric scanning from autoradiograms that were exposed 24 h was used for this purpose. Quantification of autoradiograms (Fig. 4) showed a slow increase in β-actin gene expression from 0 to 36 h, while cyclin B1 decreased in early G1 and then increased in S. This result was reproducible in four separate experiments. The up-regulation of β-actin may be related to the release of cells from G1 block and growth stimulation. Previous studies have shown an increase in the expression of other structural genes such as α-tubulin in growth-stimulated cells (37).

To investigate the role of polyamines in estrogenic regulation of cyclin B1 mRNA, we used DFMO for depleting polyamines (39). Dose- and time-dependent effects of DFMO on polyamine levels in isoleucine-starved MCF-7 cells were first documented for this purpose. Results shown in Fig. 5A demonstrated that maximal reduction of putrescine occurred with 1 mM DFMO treatment for 48 h, even though significant reduction was observed at the 24-h time point ($P < 0.05; n = 4$). At 48 h, 1 mM DFMO reduced putrescine concentrations to 10% of that in the control cells at the same time point. There were no significant differences in the reduction of putrescine between 1 and 2 mM concentrations of DFMO. The effect of DFMO on spermidine concentration was also time dependent, the maximal effect occurring only at 72–96 h (Fig. 5B). At the 48-h time point, 1 mM DFMO treatment reduced spermidine concentration to 25% of that in the control cells at the same point. In contrast to a significant reduction...
were harvested 2 h after the addition of estradiol and the change to 48 h. Cells were harvested 2 h after the addition of estradiol and the change to regular medium. Total RNA was extracted and analyzed for cyclin B1 mRNA levels as in the previous experiments. Fig. 6 shows that the intensity of cyclin B1 mRNA signal was up-regulated in DFMO-treated cells at all concentrations tested. Densitometric scanning of the autoradiograms with normalization for RNA levels in the gel indicated a 2- to 3-fold increase in cyclin B1 mRNA in the presence of DFMO compared to that of untreated cells. These increases were statistically significant ($P < 0.001; n = 4$). In separate experiments, we found that 0.1 mM DFMO had no significant effect on cyclin B1 mRNA levels (results not shown). Thus, optimal effect of DFMO on cyclin B1 mRNA was achieved at 0.5 to 1 mM concentration.

In the next set of experiments, we determined the effect of exogenous putrescine and spermidine on DFMO-mediated increase in cyclin B1 mRNA in G1 of MCF-7 cells in the presence of estradiol. Fig. 7 shows our results on the effect of polyamine repletion on cyclin B1 mRNA. Both compounds reversed the effect of DFMO on cyclin B1 mRNA stability and accelerated its degradation in a dose-dependent manner. At 100 μM putrescine, the cyclin B1 mRNA level was 10% of that in DFMO-treated cells. There was no significant change in the level of cyclin B1 mRNA at 250, 500, and 1000 μM concentrations of putrescine, compared to that at 100 μM. Spermidine at 25 μM did not affect cyclin B1 mRNA levels significantly, but at 50, 100, and 250 μM concentrations, cyclin B1 mRNA was rapidly degraded. These experiments were conducted in the presence of 1 mM aminoguanidine to prevent oxidative metabolism of polyamines by serum-derived amine oxidases (40). Treatment with aminoguanidine alone had no effect on cyclin B1 mRNA levels (results not shown). These results were reproducible in three separate experiments and confirm our hypothesis that polyamines play a major regulatory role in controlling the levels of cyclin B1 mRNA. Measurement of polyamine levels of isoleucine-starved and DFMO-treated cells showed that exogenous putrescine and spermidine were transported into the cells within 30 min of its addition to the medium, and they remained high for at least 8 h in these cells (41).

In another set of experiments, we examined the structural specificity of polyamines in cyclin B1 mRNA stability using homologues of putrescine. Isoleucine-starved cells were treated with 1 mM DFMO for 48 h and then with 100 μM C3, C4 (putrescine), C5, or C6 in the presence of aminoguanidine for 2 h. Cell cycle was then initiated by changing to regular medium containing 1 mM DFMO, 100 μM putrescine, or its homologues, 1 mM aminoguanidine and 4 μM estradiol. Cells were harvested 2 h after change to this medium; RNA was extracted and analyzed by Northern blots. Putrescine was the most effective diamine in reversing the effect of DFMO on cyclin B1 mRNA (80% reversal; $P < 0.05; n = 3$), whereas C3, C5, and C6 had no significant effect (10–25% reversal; $P > 0.1$). HPLC analysis showed that all diamines were internalized into MCF-7 cells. Thus, polyamine-induced effects on cyclin B1 mRNA are unlikely to be due to ionic effects of these molecules and indicate structure-specific interactions of polyamines in facilitating the degradation of cyclin B1 mRNA.

We also examined the effects of DFMO on cyclin B1 mRNA in the presence and absence of estradiol. Cells were treated with DFMO and 4 μM estradiol, and cyclin B1 mRNA was analyzed and quantified by slot blot experiments (autoradiogram not shown). Quantitation of slot blot signals (Fig. 8) showed a significant 3-fold increase ($P < 0.01; n = 3$) in cyclin B1 mRNA levels with 1 mM DFMO, both in the presence and absence of estradiol.

**DISCUSSION**

Our results demonstrate that estradiol plays an important role in the regulation of cyclin B1 gene expression in MCF-7 cells during the cell cycle traverse through G1→S→G2→M. In both control and estradiol-treated cells, down-regulation of cyclin B1 mRNA appears to occur immediately after the removal of the G1 cell cycle block. Within 2 h of estradiol addition, there was a 40% decrease in cyclin B1 mRNA level compared to that of untreated control cells. The cyclin B1 mRNA level in the control cultures at 8 h after the release of cell cycle block was comparable to that of estradiol-treated cells at 2 h. A similar effect of estradiol was observed on the degradation of cyclin B1 protein in G1. DFMO inhibited the degradation of cyclin B1, while the addition of exogenous putrescine and spermidine accelerated the degradation process when these compounds were added after DFMO-treatment.
Our results indicate that critical concentrations of polyamines might be necessary for the degradation of cyclin B1 and for cell cycle progression through G$_1$. These results are consistent with previous studies from our laboratory and others that suggest a polyamine pathway in estrogenic regulation of breast cancer cell growth (25-27).

Our observation of cyclin B1 mRNA in G$_1$ is comparable to other reports in the literature (17, 39). Pines and Hunter (17) conducted experiments on HeLa cells to determine if cyclin B1 mRNA levels fell during M or early G$_1$ by blocking cells in pseudo-metaphase state using a microtubule polymerization inhibitor, nocadazole. Results of those experiments indicated that cyclin B1 mRNA level remained constant until the end of M and then decreased during the initial part of G$_1$. A recent study on the expression of cyclins during liver regeneration, however, did not show mRNA for B-type cyclins in the early stages of cell cycle progression (37). On the other hand, studies on HL-60 cells showed abnormal expression of cyclins A and B1 in G$_1$, whereas such expression was not detected in immortalized normal breast epithelial cells. This result suggests a correlation of the overexpression of mitotic cyclins in G$_1$ and the expression of the malignant phenotype. It is also important to note that estradiol, a hormone that stimulates the proliferation of MCF-7 cells, accelerated the degradation of this mRNA, while DFMO, a growth inhibitory agent that arrests cells in G$_1$, attenuated its degradation.

Cyclin B1 protein is degraded in M in well-characterized embryonic model systems such as Xenopus oocytes and sea urchin eggs (44, 45). Introduction of deletion mutants of cyclin B1 protein defective in degradation leads to growth arrest in M. Thus, overexpression or lack of degradation of cyclin B1 protein is not consistent with abnormal proliferation based on the current knowledge of these events in lower organisms. However, detection of cyclin B1 protein in G$_1$ may indicate an alternate pathway by which these proteins activate cdc2-related kinases in G$_1$, bypassing the regulation of this kinase in normal cells. Nevertheless, the presence of cyclin B1 protein does not appear to be conducive to the requirements of G$_1$ transit, as cells move very slowly through G$_1$ in the absence of estradiol. Estradiol-induced enhancement of cyclin B1 degradation appears to be one of the factors facilitating cell cycle transit through G$_1$.

The induction of cyclin B1 mRNA at G$_2$/M may involve transcriptional regulation. In addition to the direct effects of estrogen receptor, these later events may include those mediated by transcription factors and DNA binding proteins induced at the early stage of estradiol action. Estradiol induction of cyclin B1 mRNA accumulation in G$_2$/M may also make a contribution from the fact that 24 h after the addition of estradiol, treated and untreated cultures are at different stages of cell cycle progression. In contrast, effects of estradiol and DFMO at the early stages of cell cycle progression could be attributed to the direct action of these agents. Accelerated degradation of cyclin B1 mRNA may contribute to estradiol-induced decrease in the duration of MCF-7 cell cycle. For example, the duration of G$_1$ of MCF-7 cells growing in the absence of phenol red and estradiol was 27 h as determined by the BrdUrd labeling method, and it was reduced to 16 h in the presence of estradiol (46, 47). Estradiol-induced decrease in the duration of G$_1$ was also reported for another estrogen-dependent breast cancer cell line, CAMA-1 (48). The 81% cell synchrony that we obtained by isoleucine starvation is comparable to other studies using purified mitotic cells. For example, Taylor et al. (49) observed 85% synchrony after mitotic selection and found that about 15% of MCF-7 cells could be categorized as noncycling cells.

The interaction between estrogen receptor and cyclin B1 mRNA might be important in its destabilization. This could be a result of the reorganization of macromolecular interactions with the ligand-induced changes in the conformation of estrogen receptor, the dissociation of heat shock proteins from the receptor, and the recruitment of transcription factors (1-3). Our previous studies have shown that polyamines increase the stability of ligand-receptor and estrogen receptor-DNA interactions in vitro (28-30). Thus, polyamines might be important cellular components that facilitate estrogenic action and the regulation of cyclin B1 accumulation and destruction during different phases of cell cycle. Since DFMO-induced increase in cyclin B1 mRNA occurred even in the absence of estradiol, polyamines may also have a direct role in the degradation of cyclin B1 mRNA.

Our finding that polyamine depletion in MCF-7 cells blocked estradiol-induced degradation of cyclin B1 mRNA indicates that cyclin B1 could be a target site at which the effect of estradiol is linked to cellular polyamine concentrations. DFMO exerted a time- and dose-dependent effect in depleting putrescine and spermidine, and the effect of DFMO on cyclin B1 mRNA was significant only after substantial reduction of these polyamine levels was achieved. Exogenous putrescine and spermidine reversed the effect of DFMO by a relatively short-term exposure of the cells to these compounds. The effect of polyamines appear to be structure-specific since putrescine homologs differing by 1 or 2 methylene groups in their chemical structure had no effect on the degradation of cyclin B1. The polyamine structural specificity that we observed in this study is comparable to polyamine effects on DNA conformational transitions and the ability of polyamine homologs to support cell growth (50-52).

We also characterized estradiol-induction of ODC activity and polyamine levels in cells synchronized by isoleucine starvation (41). The peak of ODC activity was broad starting from 8 to 16 h after addition of estradiol. Polyamine levels peaked at the 8- to 12-h time period. Thus, an increase in polyamine levels might be required for the dissociation and regrouping of protein-DNA interactions that are necessary during a rapid phase of transcription and protein synthesis that should precede DNA replication. Furthermore, polyamines are likely to be involved in the interactions between transcription factors and specific DNA sequences and in the control of mRNA stability. Our
results on the effects of DFMO and polyamines on cyclin B1 mRNA indicate that the pool of polyamines available in early G1 of the cell cycle might have an important effect in driving the cell cycle by accelerating the degradation of mitotic cyclins, such as B1, that are aberrantly expressed in this phase.

Phosphorylation levels of cyclin B1 and p34^cyc^2^k2^ are other important factors that determine the activation of cdc2 kinase activity (53, 54). Kinase inhibitors have been reported to suppress estrogen-stimulated growth of MCF-7 cells (55). Thus, a complete characterization of the role of cyclin B1 on estradiol-induced regulation of cell cycle will require the determination of concentrations of cyclin B1 mRNA, protein, its phosphorylation status, and its association with p34^cyc^2^k2^.

Recent studies demonstrate that cdc2 and homologous kinases are critical to the control of cell cycle, not only at the initiation of M but also at the onset of S (56). Estradiol may affect these processes at many different levels.

In summary, our results show a differential effect of estradiol on cyclin B1 mRNA levels during different phases of cell cycle. During early G1, estradiol facilitates the down-regulation of cyclin B1 mRNA, whereas during G2/M, it enhances the accumulation of cyclin B1 mRNA. Effect of estradiol on cyclin B1 mRNA in G1 was correlated to that on its protein. Polyamines appear to be necessary for the degradation of cyclin B1 mRNA both in the presence and absence of estradiol. The effect of estradiol on cyclin B1 in G1 may constitute a part of estrogenic action in shortening the duration of MCF-7 cell cycle and thus contribute to the hormonal regulation of cell growth.

ACKNOWLEDGMENTS

We thank Chia-Ching Chao and Carol A. Faaland for technical assistance and Dr. Edward Yurkow for flow cytometry. We thank Drs. Jonathon Pines and Tony Hunter of the Salk Institute (La Jolla, CA) for the cyclin B1 cDNA probe and Dr. Pines for a critical reading of the manuscript. We also acknowledge Marion Merrell Dow (Cincinnati, Ohio) for the supply of DFMO.

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Thresia Thomas and T. J. Thomas


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