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Differential Response of the Human Cyclin B1 Promoter to Inhibitors of the Cell Cycle in NIH3T3 Cells

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In this study, NIH3T3 cells stably transfected with a cyclin B1-luciferase reporter vector were utilized to investigate if cyclin B1 promoter activity is linked to either DNA replication or the activities of various cyclin-cyclin dependent kinases (cdks). Synchronized cells treated at the time of serum re-stimulation with 2 μg/ml of the DNA synthesis inhibitor, aphidicolin, did not display an increase in luciferase activity in comparison to control cells. When treated with aphidicolin during S phase, luciferase activity decreased. In contrast, luciferase activity increased in cells treated at the time of serum re-stimulation with 200 μM olomoucine, a cyclin-cdk inhibitor. These results indicate that (1) cyclin B1 promoter activity in NIH3T3 cells is linked to a DNA replication checkpoint control mechanism; (2) the cyclin B1 gene can be activated in the absence of functional cyclin E-cdk2, cyclin A-cdk2, or cyclin B-cdk2; and (3) cyclin B1 gene activation can occur in G1 arrested cells under conditions in which the arrest is not directly linked to inhibition of DNA synthesis.

Cell cycle progression is controlled by a group of proteins called cyclins. These cyclins complex with cyclin dependent kinases (cdks) and phosphorylate a variety of substrates, which are directly involved in the events of the cell cycle (1,2,3). Different cyclin-cdks function at different cell cycle periods. Cyclin D-cdk4 (or cdk6), cyclin E-cdk2, and cyclin A-cdk2 act in this order to control progression from G1 into S phase and DNA synthesis. The cyclin B-cdk1 complex, which forms in late S/G2, is then responsible for entry into mitosis. The cell utilizes a variety of mechanisms to ensure that the cyclin-cdks are sequentially activated in this orderly fashion. One of these mechanisms is the regulated appearance of the cyclins by transcriptional activation of the cyclin genes.

The focus of our investigations has been the transcriptional regulation of the human cyclin B1 gene. By mutational analyses, we showed that 287 bp of the cyclin B1 upstream sequence is sufficient for S phase activation and that the two CCAAT boxes located within this region are essential for expression (4). We also demonstrated that the cyclin B1 gene can be transactivated by overexpressed cyclin D-cdk4, cyclin A-cdk2, and cyclin E-cdk2. In other studies, cyclin B transcription was found to be reduced in cells treated with various DNA damaging agents (5, 6). These findings indicate that the cyclin B1 promoter may be subject to multiple levels of regulation, linking its activity to upstream cell cycle events via the cyclin-cdks and various checkpoint controls, including DNA damage.

In this study, we took advantage of our cyclin B1-luciferase reporter system to examine the relationship of cyclin B1 promoter activity to other cell cycle events, using two different inhibitors of the cell cycle. Aphidicolin, which directly inhibits DNA synthesis by binding to DNA polymerase α, δ, and ϵ (7) was used to determine if cyclin B1 transcription is linked to DNA replication in NIH3T3 cells. The involvement of other cyclin-cdks in cyclin B promoter activity was explored with the drug olomoucine, which inhibits the activities of cyclin A-cdk2, cyclin E-cdk2 and cyclin B-cdk1 (8).

METHODS

Cell culture. NIH3T3 cells (termed 3T3-hcycB287LUC) that had been stably transfected with the cyclin B-luciferase reporter vector hcycB287LUC (4) were grown in DMEM-C, composed of Dulbecco's Modified Eagle's Medium (DMEM) containing 1% v/v penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin), 0.2% v/v of 250 mg/ml amphotericin B, and 10% calf serum (Sigma).

Inhibitor studies. 3T3-hcycB287LUC cells were plated in either 6 or 24 well dishes at a cell density of 1×10^5 or 1.8×10^4 cells per well, respectively. The cells were synchronized by serum starvation for 60 hours by growing the cells in the same DMEM medium de-

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scribed above but with 0.5% calf serum. At time zero, growth was re-initiated by changing the medium to DMEM-C. Olomoucine (BIOMOL) was prepared as a 100 mM stock in dimethylsulfoxide (DMSO). Aphidicolin (Sigma) was prepared as a 1 mg/ml stock in DMSO. Cells were treated with olomoucine by addition of the appropriate amount of the 100 mM stock solution to the medium to give a final concentration of 200 μM. The equivalent amount of DMSO was added to the control wells (without olomoucine). Aphidicolin stock was added to give a final concentration of 2 μg/ml. At selected times, cells were collected from individual wells by removing the medium and washing the cells one time with Ca^{2+}/Mg^{2+} free Hank's solution. One hundred microliters of Cell Culture Lysis Reagent (Promega) diluted 1:5 in water was added to each well and the plates incubated for an additional 15 minutes at 37°C. Each well was then scraped and the lysate collected.

Tritiated thymidine incorporation. To determine the period of DNA synthesis, 0.5 mCi/ml of ^{3}H-thymidine (specific activity, 46 Ci/mmol) was added per well, one half hour prior to collecting the cells. Counts incorporated were determined from aliquots of the cell lysates by trichloroacetic acid precipitation and filtration onto glass fiber filters (9).

Luciferase assays. Twenty or thirty microliter aliquots of the cell lysates were directly assayed in a Berthold Lumat LB9501 luminometer using the Luciferase Assay Kit (Promega). Activity was measured as relative light units (RLUs).

FACS analysis. Cells were prepared for FACS analysis by first removing the medium from the appropriate wells and washing the cells twice with Ca^{2+}/Mg^{2+} free Hanks. Trypsin solution (0.25% porcine trypsin/0.02% EDTA) was added for a period of one minute to release the cells. An equal volume of DMEM-C was added and the cells pelleted by centrifugation. The resulting cell pellet was washed one time in Ca^{2+}/Mg^{2+} free Hanks and then resuspended in 70% methanol diluted in Ca^{2+}/Mg^{2+} free Hanks. On the day of FACS, the fixed cells were pelleted and resuspended in 0.1% Triton X-100, 50 μg/ml RNAase A, 20 μg/ml propidium iodide and incubated for at least 10 minutes at room temperature. The cells were analyzed on a Becton Dickinson FACScan.

RESULTS

Initially, the effects of olomoucine and aphidicolin on cell cycle profiles were determined. 3T3-hcycB287LUC cells were synchronized in G0 by serum starvation and re-initiated into the cell cycle by the addition of 10% calf serum. Cells were treated at the time of serum stimulation with either 200 μM of olomoucine, 2 μg/ml of aphidicolin or no treatment. Twenty four hours later the cells were collected and analyzed by FACS to determine the distribution of the cells in the various phases of the cell cycle. Aphidicolin and olomoucine increased the proportion of the cells in G1 to 88% and 84%, respectively, compared to 45% for the untreated cells (Table 1). These results confirm that treatment with olomoucine or aphidicolin cause the majority of the cells to remain arrested at 24 hours as 2N cells.

We next determined the pattern of luciferase activity in 3T3-hycB287LUC cells that were treated with aphidicolin to determine if cyclin B1 promoter activity is linked to DNA replication. These NIH3T3 cells are stably transfected with the reporter construct hcycB287LUC, containing 287 bp of the human cyclin B1 promoter fused to the firefly luciferase gene (4). When

TABLE 1

FACS Analysis at 24 Hours

	%G1	%S	%G2
Control	45	21	35
Aphidicolin	88	5	7
Olomoucine	84	8	8

Note. Treatment of the cells and FACS analysis were performed as described in the Methods section.

aphidicolin was added at the time of serum re-stimulation, the level of luciferase activity remained low and did not increase as seen in the untreated, control cells (Fig. 1a). The ratio of luciferase activity between 0 and 22 hours was determined to be 6 fold, compared to 28 fold value for the untreated controls (Table 2). Only a low level of DNA synthesis was detected in the aphidicolin treated cells based on incorporation of tritiated thymidine (Fig. 2).

When aphidicolin is added to cells at the time of serum re-stimulation, the drug is present during the initiation process of DNA synthesis. To determine if cyclin B1 promoter activity is linked to on-going DNA synthesis, aphidicolin was added to synchronized cells, 18 hours after serum re-stimulation. By 18 hours in control cells, DNA synthesis can be detected by incorporation of tritiated thymidine and the level of luciferase activity starts to increase (4). Addition of aphidicolin at 18 hours resulted in an immediate decrease in tritiated thymidine incorporation, while incorporation in the control cells continued to increase (data not shown). FACS analysis of the aphidicolin treated cells at 24 hr indicated that only 19% of the cells were allowed to pass into G2 phase while 42% of the control cells were in G2 (data not shown). A drop in luciferase activity was detected in aphidicolin treated cells, which lagged approximately 4 hours behind the detected decrease in DNA synthesis (Fig. 1b). This lag is likely due to the three hour half life of the luciferase protein (10). By 22 hours, luciferase activity in the aphidicolin treated cells was only 13 fold higher than its time 0 amount, compared to 28-fold for the untreated cells (Table 2).

Luciferase activity was then measured in cells treated with olomoucine to determine if cyclin B1 promoter activation requires the activity of cyclin E-cdk2, cyclin A-cdk2 or cyclin B-cdk1. During the released from serum starvation, cells were either untreated or treated with 200 μg/ml olomoucine. The cells were collected at various time intervals over a period of 24 hours and assayed for luciferase activity. The pattern of luciferase activity in control and olomoucine treated cells is similar up to approximately 23 hours (Fig. 3a). The increase in luciferase activity between 0 and 22 hours for the olomoucine treated cells was found to be, on average 32 fold, compared to a value of 28 for the

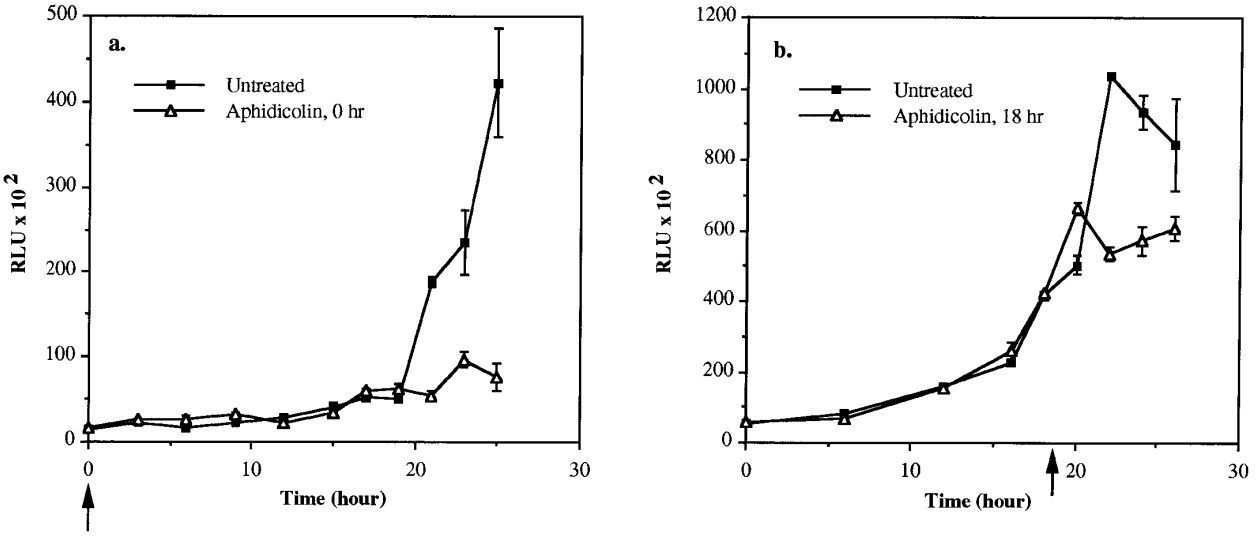

FIG. 1. Effect of aphidicolin on luciferase activity. (a) 3T3-hcycB287LUC cells were synchronized as described in the Methods section and treated with 2 μg/ml aphidicolin at time zero (arrow) after re-initiation of the cell cycle. Cells were collected at the indicated times and assayed for luciferase activity, which is expressed as relative light units (RLU). (b) Same as in "a" but aphidicolin was added at 18 hours (arrow) after re initiation of the cell cycle. Shown are representative graphs from two (b) or three (a) independent experiments. Bars indicate the standard error derived from two (b) or four (a) individual determinations per time point.

control (Table 2). This increase occurred in cells whose DNA content was predominantly 2N (Table 1) and only a low level of DNA synthesis could be detected by tritiated thymidine incorporation (Fig. 2).

It appears that the level of luciferase activity in olomoucine treated cells may reach a maximum after 23 hours and possibly decrease or plateau. To explore this issue, luciferase activity was measured in control and olomoucine treated cells at later time points. As seen in Fig. 3b, luciferase activity in cells treated with olomoucine at the time of serum re-stimulation increased in parallel with the controls up to 22 hours and then leveled out before increasing slightly by 30 hours. These results indicate that cyclin B1 promoter activity does, indeed, increase in olomoucine treated cells but a steady state level is reached after more than 22 hours of treatment. In contrast, luciferase levels continued to rise after 22 hours in the untreated cells.

TABLE 2

Fold Change in Luciferase Activity

	T22/T0	
Control	28.2 ± 1.6	n = 4
Aphidicolin		
0 hour	6.3 ± 0.8	n = 4
18 hour	12.6 ± 0.4	n = 2
Olomoucine	37.1 ± 10.3	n = 3

Note. Values of RLU at time 0 hour (T0) and 22 hour (T22) were used to determine fold increase. Aphidicolin was added at 0 and 18 hour; olomoucine at 0 hour. The averages of the indicated number (n) of determinations are shown, plus and minus standard error.

DISCUSSION

In this study, we demonstrated that cyclin B1 promoter activity is linked to DNA synthesis in NIH3T3 cells. In addition, we found that the cyclin B1 promoter

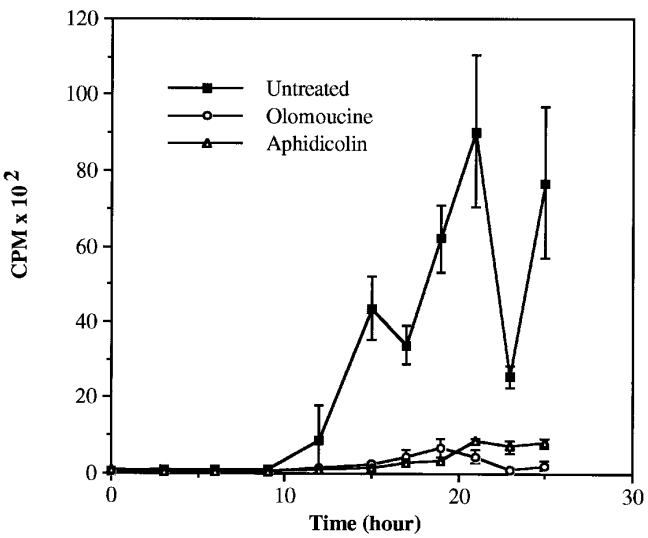

FIG. 2. Effect of olomoucine and aphidicolin on DNA synthesis. 3T3-hcycB287LUC cells were synchronized as described in the Methods section and treated with olomoucine, aphidicolin or left untreated at the time of re initiation into the cell cycle. Counts incorporated of ^{3}H-thymidine were determined for each time point. These data were derived from the same cells used to generate Figures 1a and 3a. Very little DNA synthesis was detected in the olomoucine and aphidicolin treated cells in comparison to the untreated, controls.

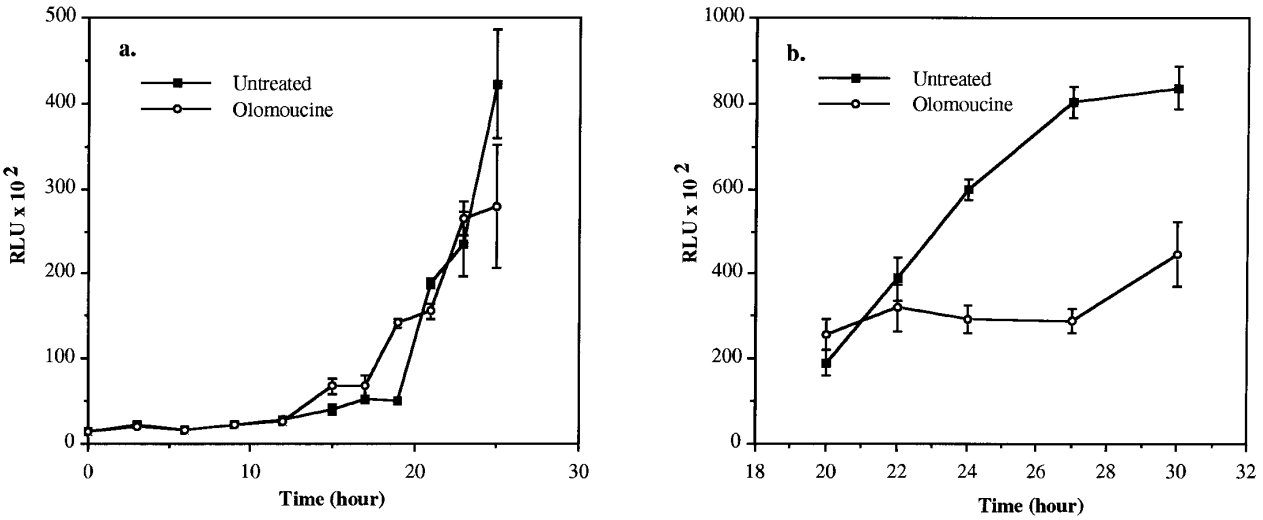

FIG. 3. Effect of olomoucine on luciferase activity. (a) 3T3-hcycB287LUC cells were synchronized as described in the Methods section and left untreated or made 200 μM olomoucine at time zero after re initiation of the cell cycle. Cells were assayed for luciferase activity (expressed as RLU) at the indicated times. (b) Same as "a" but luciferase activity was determined at later time points, following re initiation of the cell cycle. Shown are representative graphs from two (b) or three (a) independent experiments. Bars indicate standard error derived from four (a) or five (b) individual determinations per time point.

can be activated in G1 arrested cells in the absence of cyclin E-cdk2, cyclin A-cdk2 and cyclin B-cdk1 kinase activities. Taken together, these results suggest that cyclin B1 gene transcription is subject to a DNA replication checkpoint control mechanism and that the cyclin B promoter can be inappropriately activated in G1 arrested cells, when the G1 arrest does not involve direct inhibition of DNA synthesis.

Checkpoint control is a central feature of the cell cycle, ensuring that critical events such as DNA synthesis, DNA repair and chromosome segregation are completed with precision prior to cell division. In response to blocks in DNA replication (and DNA damage) cells become arrested during G1, S or G2. Genetic studies conducted in yeast indicate that replication checkpoint control is dependent on components of the DNA replication complex, itself, and not simply unreplicated DNA (11, 12, 13, 14). Acting as sensors, these replication complex proteins are believed to initiate a signal transduction pathway, resulting in cell cycle arrest. Cells exposed to aphidicolin in G1 are probably arrested in very early S phase with a replication complex in place, although by FACS analysis they are characterized as 2N, G1 cells. During S phase, aphidicolin inhibits on going DNA synthesis by binding to and interferring with the activity of the DNA polymerase. Consequently, aphidicolin treatment in G1 and S should initiate a complex series of events leading to checkpoint control. Our data suggests that one component of this checkpoint control mechanism in NIH3T3 cells is downregulation of cyclin B1 gene transcription. This decrease would contribute to a G2 arrest by causing a reduction in cyclin B protein, which is required for entry into mitosis. Removal of the aphidicolin from the cells should allow for resumption of DNA synthesis, followed by re-initiation of cyclin B1 transcription. This is, indeed, what we have found (data not shown).

In olomoucine treated cells, cyclin E-cdk2, cyclin A-cdk2, and cyclin B-cdk1 are inhibited (8). Cyclin E-cdk2 is required for passage of the cell from G1 into S phase and also for a feedback control mechanism, downregulating cyclin D expression (15, 16, 17). Inhibition of cyclin E-cdk2 activity blocks entry into S, leaving the cell in an extended G1 phase (18). According to our data, functional cyclin E-cdk2, cyclin A-cdk2 and cyclin B-cdk1 are not required for activating the cyclin B1 promoter. It is likely, however, that the activities of some of these cyclin-cdks are involved in establishing the normal timing and appropriate level of cyclin B1 promoter activation in S/G2. One likely explanation for the olomoucine induced activation of cyclin B1 transcription is that the cyclin B1 promoter is responding to increased activity of cyclin D-cdk4 (cdk6), which may be elevated in olomoucine treated cells due to lack of inhibition by cyclin E-cdk2. Support for this hypothesis is our demonstration that overexpression of cyclin D-cdk4 can result in transactivation of the cyclin B1 promoter (4). In addition, cyclin B has been shown to be overexpressed in a variety of cancer cell lines with increased cyclin D expression (19, 20). Since an S phase DNA initiation complex is probably absence in olomoucine/G1 arrested cells, the cyclin B1 gene would not be subject to replication checkpoint control and be capable of responding to olomoucine induced alterations in the G1 cells.

The response of the cyclin B1 promoter to inhibition

of DNA replication can be mapped to particular sequences within the promoter by mutational analysis. It is likely that the CCAAT sequences required for normal, cell cycle activation of the cyclin B1 promoter (4) are also required for DNA replication checkpoint control. It is also reasonable to hypothesize that these same sequences are involved in the G1 activation of the cyclin B1 promoter in olomoucine treated cells. We are currently in the process of examining these possibilities.

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