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Mode of Estrogen Action on Cell Proliferation in CAMA-1 Cells: II. Sensitivity of G1 Phase Population

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The mammary cancer cell line CAMA-1 synchronized at the G1/S boundary by thymidine block or at the G1/M boundary by nocodazole was used to evaluate 1) the sensitivity of a specific cell cycle phase or phases to 17β-estradiol (E_2), 2) the effect of E_2 on cell cycle kinetics, and 3) the resultant E_2 effect on cell proliferation. In synchronized G1/S cells, E_2-induced ^{3}H-thymidine uptake, which indicated a newly formed S population, was observed only when E_2 was added during, but not after, thymidine synchronization. Synchronized G2/M cells, enriched by Percoll gradient centrifugation to approximately 90% mitotic cells, responded to E_2 added immediately following selection; the total E_2-treated population traversed the cycle faster and reached S phase approximately 4 hr earlier than cells not exposed to E_2. When E_2 was added during the last hour of synchronization (ie, at late G2 or G2/M), or for 1 hr during mitotic cell enrichment, a mixed response occurred: a small portion had an accelerated G1 exit, while the majority of cells behaved the same as controls not incubated with E_2. When E_2 addition was delayed until 2 hr, 7 hr, or 12 hr following cell selection, to allow many early G1 phase cells to miss E_2 exposure, the response to E_2 was again mixed. When E_2 was added during the 16 hr of nocodazole synchronization, when cells were largely at S or possibly at early G2, it inhibited entry into S phase. The E_2-induced increase or decrease of S phase cells in the nocodazole experiments also showed corresponding changes in mitotic index and cell number. These results showed that 1) the early G1 phase and possibly the G2/M phase are sensitive to E_2 stimulation, late G1, G1/S, or G2 are refractory; 2) the E_2 stimulation of cell proliferation is due primarily to an increased proportion of G1 cells that traverse the cell cycle and a shortened G1 period, 3) E_2 does not facilitate faster cell division; and 4) estrogen-induced cell proliferation or G1/S transition occurs only when very early G1 phase cells are exposed to estrogen. These results are consistent with the constant transition probability hypothesis, that is, E_2 alters the probability of cells entering into DNA synthesis without significantly affecting the duration of other cell cycle phases. Results from this study provide new information for further studies aimed at elucidating E_2-modulated G1 events related to tumor growth.

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Abundant evidence shows that the growth of mammary carcinoma cells is affected by many hormones, both in vivo and in vitro [1]. Employing an in vitro model with the human mammary adenocarcinoma cell line CAMA-1, we demonstrated that 17β-estradiol (E_2) stimulates cell proliferation in a dose-related manner [2,3]. The E_2-induced cell growth requires the presence of serum [2,4]. We reported that E_2 increases cell yield, shortens the population doubling time [2], and increases other biochemical parameters [5,6] in an asynchronous population. Others [7–11] have also demonstrated that E_2 enhances G1 to S phase transit in tissues and asynchronized cells. Knowledge of the exact point in the cell cycle at which estrogen exerts its effect and detailed cell cycle kinetic studies with synchronized populations are lacking. This report aims to evaluate these areas. Two procedures were developed to synchronize CAMA-1 cells, one at the G1/S boundary by thymidine (dThd) block and the other at the G2/M phase by nocodazole treatment. Synchronized cell populations were then tested for their sensitivity to E_2 and for the effect of E_2 on the proliferative kinetics.

MATERIALS AND METHODS

Cells and Tissue Culture

CAMA-1 (a gift from Dr. J. Fogh) is a human breast cancer cell line originating from the malignant pleural effusion of a postmenopausal woman with adenocarcinoma of the breast [12]. Cells were routinely maintained in minimal essential medium (MEM) containing 25% fetal bovine serum (FBS) (Biologos, Napierville, IL), 100 units of penicillin-streptomycin, and 2 mM L-glutamine with weekly passages as described previously [2]. For experiments, cells were grown in the same medium with 10% dextran-charcoal-treated FBS (DCFBS) in place of 25% FBS. Steroids were dissolved in 95% ethanol before adding to medium to give a final ethanol concentration of 0.1% or less. When grown routinely, CAMA-1 cells have a population doubling time of 2 to 2 ½ days.

Cell Synchronization

To synchronize at the G1/S boundary, cells were plated in medium containing 3 mM dThd for 40 hr, at which time the dThd was removed, the plate was rinsed once, and fresh medium was added (time 0). Estrogen (1 nM) was added during or after synchronization as described in "Results."

To obtain a population of cells at G2/M, CAMA-1 cells were heavily plated in 10% DCFBS for 5 days, by which time they had reached the stationary phase. The medium was replaced with fresh 10% DCFBS for 20 hr followed by the addition of 0.05 μg/ml nocodazole, methyl [5-(2 thienyl-carbonyl) 1H-benzimidazole-2-yl] carbamate, R17934NSC, from Sigma Chemical Co. (St. Louis, MO) which effectively blocked cells at the G2/M boundary [13]. Loosely attached mitotic cells were readily released from the monolayer by shaking. The released cells were further purified by sedimentation on a discontinuous Percoll (Pharmacia, Inc. Piscataway, NJ) gradient yielding a 95% viable population containing approximately 90% mitotic cells. These cells were then plated in medium appropriate for the experiment.

^{3}H-Thymidine uptake was determined by plating 5×10^4 cells in 2 ml medium per well of a six-well multiplate (Falcon) as described previously [3]. Cells were harvested by trypsin-ethylenediamine tetraacetic acid (EDTA) after a 1-hr pulse with 0.1 μCi/ml ^{3}H-dThd. After counting in a Coulter counter (model ZBI), the remaining cells were collected by filtration on glass fiber filters (Whatman 934H), dried, and counted in BudgetSolve (RPI) using a scintillation counter (Beckman LS2800) with a counting efficiency of 40% for tritium. The results were expressed as DPM per 1,000 cells to correct for fluctuations of ^{3}H-dThd uptake owing to variations in cell number per well at plating or as a result of cell growth, and the Student t-test was used for statistical analysis. That whole-cell ^{3}H-dThd uptake represents incorporation into DNA and that the increase in uptake parallels cell growth have been previously established [3].

Mitotic Index

To determine the mitotic index, cultures plated in parallel with cells for ^{3}H-dThd uptake were harvested with trypsin-EDTA, sedimented, and immediately treated with Carnoy's fixative. Mitotic figures were detected using aceto-orcein stain [14]. At least 2,000 cells were counted for each sample.

RESULTS

The effect of dThd block on cells at the boundary of G1/S phases is well documented [15]. Sensitivity of CAMA-1 cells to dThd block was tested in order to select the most effective concentration for synchronization. At 3 mM, dThd blocks proliferation of CAMA-1 cells with minimal toxicity, and a 40-hr exposure produces maximum synchrony (result not shown). Cells synchronized at the G1/S boundary in this manner rapidly progressed into S phase when the block was removed, as shown in Figure 1. In this experiment, the presence of E_2 during the 40-hr synchronization process stimulated a slight but significant increase in ^{3}H-dThd uptake in the first S phase. Thereafter, no difference in ^{3}H-dThd uptake was noted between E_2-treated and untreated cells. This effect of E_2 was repeatable in other experiments in which cells were synchronized by the 40-hr dThd block, but it was not noted if the dThd block with E_2 was conducted for less than one-third of a generation time (ie, less than G1 duration). These results showed that E_2-induced ^{3}H-dThd uptake, ie, S phase formation, is attributed to E_2 stimulation of cells before they reached the G1/S period. In this experiment, only a small number of cells had divided by 25 hr after medium change, and there was no difference between E_2-treated and untreated cells (Fig. 1, bottom lines). Conversely, when cells were synchronized in the absence of E_2 and then incubated in fresh medium with E_2 present or absent, no E_2-induced ^{3}H-dThd uptake was observed in the first S phase, while uptake during the second S phase was significantly higher ($P < .01$) in the presence of E_2 during the period from 30 to 50 hr following medium change (Fig. 2A). This result is consistent with previous experiments showing that E_2 modulates events that occur before DNA synthesis. Furthermore, by virtue of the failure to observe stimulation by E_2 in the first S peak, the late G1, the G1/S, or the period immediately following is insensitive to E_2 stimulation. Partial synchronization of cells by a 16-hr dThd block yielded a similar E_2-stimulated second peak (Fig. 2B), which occurred earlier than when cells were fully synchronized. This is not surprising since exposure of cells to dThd for 16 hr

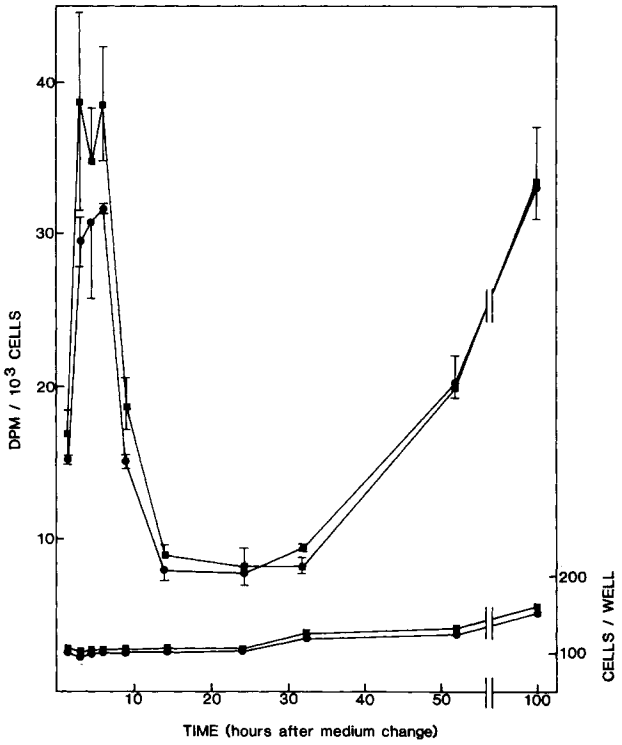


Fig. 1. Uptake of ^{3}H-dThd and proliferation of cells synchronized in 10% DCFBS with dThd (3 mM) as described in the text. Estrogen was present (■) or absent (●) during the synchronization process (40 hr) and removed at the medium change (time 0). Each point is the mean ± SD of triplicates. At 6 hr $P < .05$, at 9 hr $P < .025$, for E_2-treated vs control.

(approximately 1/3 of a generation) would block only a portion of the total cell population at the G1/S boundary, and the other portion of cells that did not reach G1/S phase would do so after medium change and E_2 exposure (time 0). Blocked cell population readily entered S phase following selection, as shown by the first ^{3}H-dThd peak, and these cells did not respond to E_2 stimulation, showing that E_2 action on induced proliferation is not exerted at the cycle immediately following G1/S period. In contrast, unblocked cells that now had a chance to progress through G1 phase during E_2 exposure exhibited a marked increase in ^{3}H-dThd incorporation over controls not exposed to E_2; the increased uptake was noted commencing at approximately 20 hr, reaching a maximum at approximately 30 hr. By virture of these cells being nonsynchronized, this peak was much broader than the first peak. Since the mean G1 phase duration of cells not treated with E_2 was approximately 28 hr (see Fig. 5A), this broad peak might best describe cells at the early G1 phase that responded to E_2 stimulation. This experiment is consistent with the notion that E_2-mediated events that modify the cell cycle transit time and facilitate an increase of S phase entrance are present at the G1 phase. Taken collectively (Figs. 1, 2), these results show that the E_2-induced ^{3}H-dThd uptake observed was not dependent on the presence of E_2 during the S phase. Similarly, E_2 exerted no immediate effect on cells at the late G1 phase, the G1/S boundary, or the period immediately after.

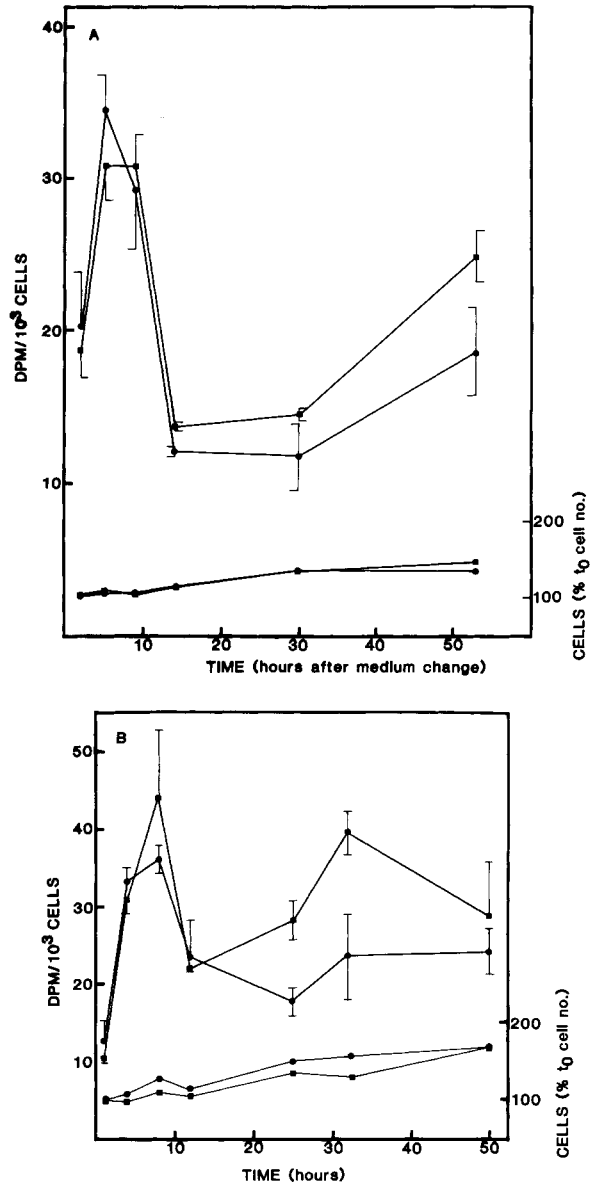


Fig. 2. Uptake of ^{3}H-dThd and proliferation of cells synchronized in 10% DCFBS with dThd for 40 hr (**A**) and 16 hr (**B**). Estrogen was added at time of medium change. (●), Control; (■), E_2. For uptake at 53 hr (A) $P < .01$ for E_2-treated vs control.

To exclude artifacts that might arise from different experimental conditions, the effect of E_2 on the induction of ^{3}H-dThd uptake during the first S phase was reexamined in a single experiment under the above two conditions, ie, addition of E_2 before and after synchronization. As shown in Figure 3, a significant increase ($P < .01$) in ^{3}H-dThd uptake was achieved only when E_2 was added during the time of dThd synchronization, during which G1 phase cells would be exposed to E_2. During G1/S or S phases, cells appear to be insensitive to E_2 stimulation. Tests were then

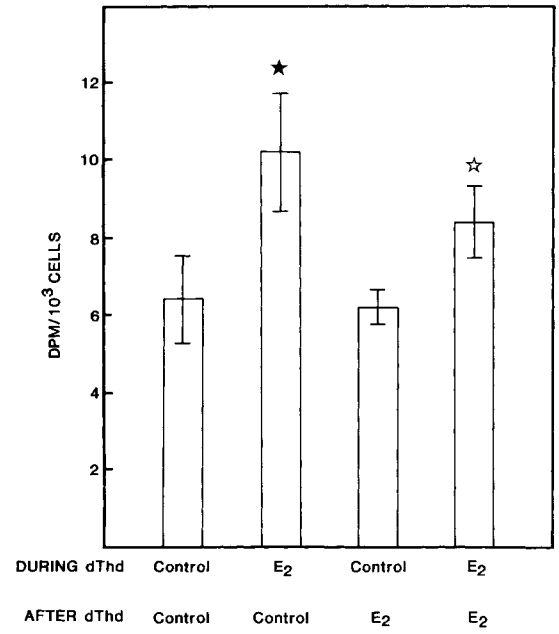


Fig. 3. Uptake of ^{3}H-dThd during the first S-phase following dThd synchronization. Estrogen was added during or after synchronization or at both times as indicated. Each bar represents the mean ± SD (n = 4). $P < .01$ (★) and .05 (☆) vs E_2-free control.

performed to determine which of the specific periods, ie, early, mid-, or late G1, G2, or M phase, was sensitive to E_2 stimulation.

To examine the E_2 effect on early G1 phase, we synchronized cells at the G2/M boundary with nocodazole. The most effective dose with minimal cytotoxicity was found to be 0.05 μg/ml. Following synchronization, a mixture of mitotic cells and G1 phase cells were released by gentle shaking, yielding about 50–70% mitotic cells. Since G2/M cells contain twice as much low-density nucleic acid, it seemed plausible that separation by density could be achieved. As shown in Figure 4, this was indeed accomplished with the added benefit that dead cells did not penetrate the Percoll gradient and could be easily discarded. The resulting nearly homogeneous mitotic population in 1.04-density Percoll could then be plated for ^{3}H-dThd uptake studies.

The effect of E_2 on different cell cycle phases was tested in three experiments. In experiment 1, G1 phase cells treated with E_2 after selection were clearly different from untreated cells (Fig. 5A), and the differences in ^{3}H-dThd uptake at all the time points from 20 to 30 hr were highly significant ($P < .001$). The entire population of E_2-treated cells (CCE) traversed the cycle faster and reached the first S phase approximately 4 hr earlier than cells receiving no exposure to E_2 (CCC). Under this experimental condition, the mean G1 phase in untreated cells was approximately 28 hr; E_2 shortened the G1 duration to 24 hr without an apparent effect on the duration of the S or G2 phases. A distinct second ^{3}H-dThd peak was noted with E_2-treated cells at about 62 hr, but was not apparent in untreated cells. It is not clear whether this peak was derived from the cell population of the first peak or from the asynchronized population (approximately 10% G1 phase cells were still mixed with G2/M synchronized cells).

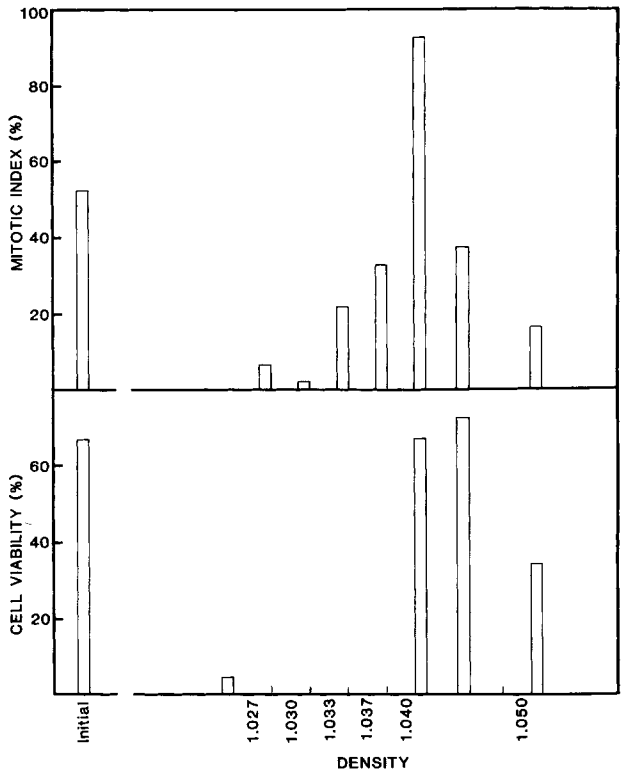
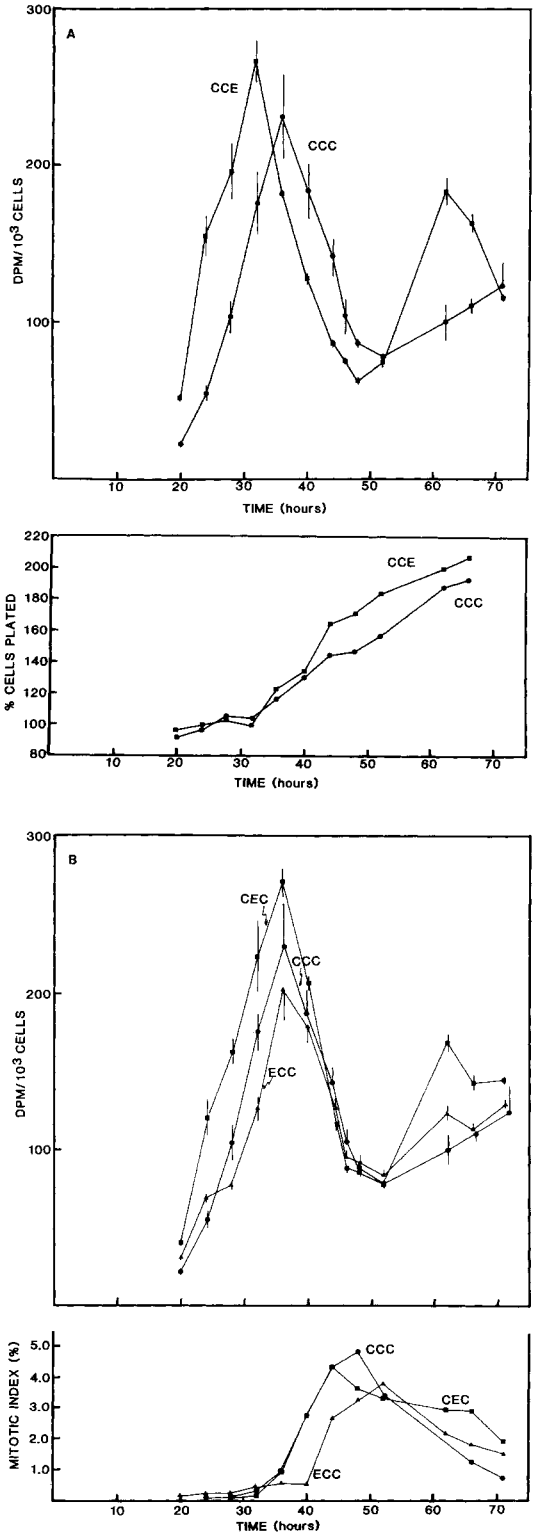


Fig. 4. Purification of G2/M cells using Percoll density gradient. Note that the lightest cells were virtually all nonviable, while cells at density 1.040 were largely mitotic and viable.

The effect of E_2 on ^{3}H-dThd uptake is supported by an increase in cell number in the E_2-treated population ($P < .01$) and was noticeable after E_2 treatment (Fig. 5A, lower panel). These results demonstrated that G1 cells are sensitive to E_2, which stimulates cell proliferation by increasing the proportion of cells progressing through the cell cycle and shortening the duration of the G1 phase. The net result is an increase in the number of dividing cells at the end of each cell generation with an accelerated cycling time during the G1/S transition.

Experiments 2 and 3 (Fig. 5B), done in parallel with experiment 1, were designed to test the sensitivity of other cell phases to E_2. When E_2 was added only during the last hour of nocodazole treatment (CEC), when cells were in G2/M, it was observed that CEC cells had a higher and wider S peak, partly owing to a portion of cells incorporating ^{3}H-dThd earlier, while most of the cells behaved no differently from cells not treated with E_2 (CCC). The earlier ^{3}H-dThd incorporation was similar to that observed in Figure 5A for CCE-treated cells, in which a second ^{3}H-dThd peak was also noted. The increase in ^{3}H-dThd was not accompanied by an increase in mitotic index (Fig. 5B, lower panel) or cell number (not shown) during the first 45 hr following cell selection. This finding shows that E_2 action under CEC-treatment does not hasten cell division. Similarly to CCE-treatment (Fig. 5A), E_2 enhances the progression of the G1 phase to the S phase. Owing to the fact that a small portion of early G1 phase cells were present in this preparation (data of flow cytometric analysis


A
CCE
CCC
DPM/10³ CELLS
TIME (hours)
% CELLS PLATED
B
CEC
CCC
ECC
MITOTIC INDEX (%)

not shown), further investigation is required to ascertain the effect E_2 may have on the G2/M phase.

In experiment 3, during the process of a 16-hr nocodazole synchronization, the progress of S and G2 cells is blocked at the G2/M boundary. Exposure of cells to E_2 during synchronization (ECC) caused a slight delay in ^{3}H-dThd uptake as compared with CEC, CCE, and CCC treatments (Fig. 5B, upper panel). A small portion of these cells (shoulder of the first S peak) exhibited behavior similar to control cells (CCC) not subjected to E_2 treatment. The delay in ^{3}H-dThd uptake was further revealed by a delay in the appearance of mitotic figures (Fig. 5B, lower panel). This inhibitory action of E_2 is quite unexpected; this effect, however, should be documented through additional experimentation.

The foregoing experiments collectively demonstrate that either the G2/M boundary or the very early G1 phase is most likely the period in which E_2 exerts its stimulatory effect on cell proliferation. Experiments were then designed to investigate whether G1 phase cells at different levels of maturity are also sensitive to E_2 stimulation. This was done by temporal addition of E_2 following selection of synchronized cells. When E_2 was added at plating, it stimulated an earlier appearing and higher S peak than in untreated cells (data not shown), consistent with previous experiments (shown in Fig. 5A). When E_2 was added to cells during G2/M phase for 1 hr, ^{3}H-dThd uptake relative to control cells without E_2 (results not shown) was similar to the description in Figure 5B, curve CEC. However, a mixed response of the cells to E_2 was noted when E_2 addition was delayed 2 hr, or 7 hr following cell selection, when an increasing portion of the G2/M phase cells have passed mitosis and the early G1 stage (Fig. 6A,B). The cells exposed to E_2 in early G1 phase responded readily, as revealed by the earlier uptake of radioactivity at about 25 hr. The portion of cells that progressed beyond the early G1 phase did not respond and were not different from cells not treated with E_2, as shown by the radioactivity profile between 30 and 48 hr. These results are consistent with those of previous experiments (Fig. 5B), which demonstrated that most cells in this preparation are not sensitive to E_2 stimulation. In all these experiments, an earlier appearance of ^{3}H-dThd uptake was always followed by an earlier appearance of mitotic figures in comparison with control cells (Fig. 6A,B, lower panels). Thereafter, the mitotic index was not different from that of control cells. Furthermore, with cells characterized by an earlier progression of G1 to S phase owing to E_2 stimulation, a second ^{3}H-dThd peak was observed. When synchronized cells were exposed to E_2 12 hr after selection, by which time many cells have reached mid- to late G1 phase, E_2 elicited a similar effect to that found in cells exposed to E_2 for 7 hr (result not shown).

Fig. 5. Uptake of ^{3}H-dThd (upper panel) and cell proliferation or mitotic index (lower panel) of CAMA-1 cells following removal of nocodazole. Cells were treated with E_2 for limited times before, during, or after Percoll enrichment. **(A)** Cells received E_2 after enrichment (CCE, ■) or not at all (CCC, ●). Each point shown is the mean of triplicates, with SD shown as bars. Time points between 20 and 30 hr for ^{3}H-dThd uptake are highly significant ($P < .001$). **(B)** Cells received E_2 for 1 hr during enrichment (CEC, ■), for 16 hr before enrichment (ECC, ▲), or not at all (CCC, ●). E_2-induced ^{3}H-dThd uptake for CEC and inhibition for ECC between 25 and 35 hr are statistically significant ($P < .01$). Experiments A and B were run concurrently. Variations of cell count for A or mitotic index for B are within 5%. E_2 treatment increased cell number significantly after 45 hr of plating ($P < .01$).

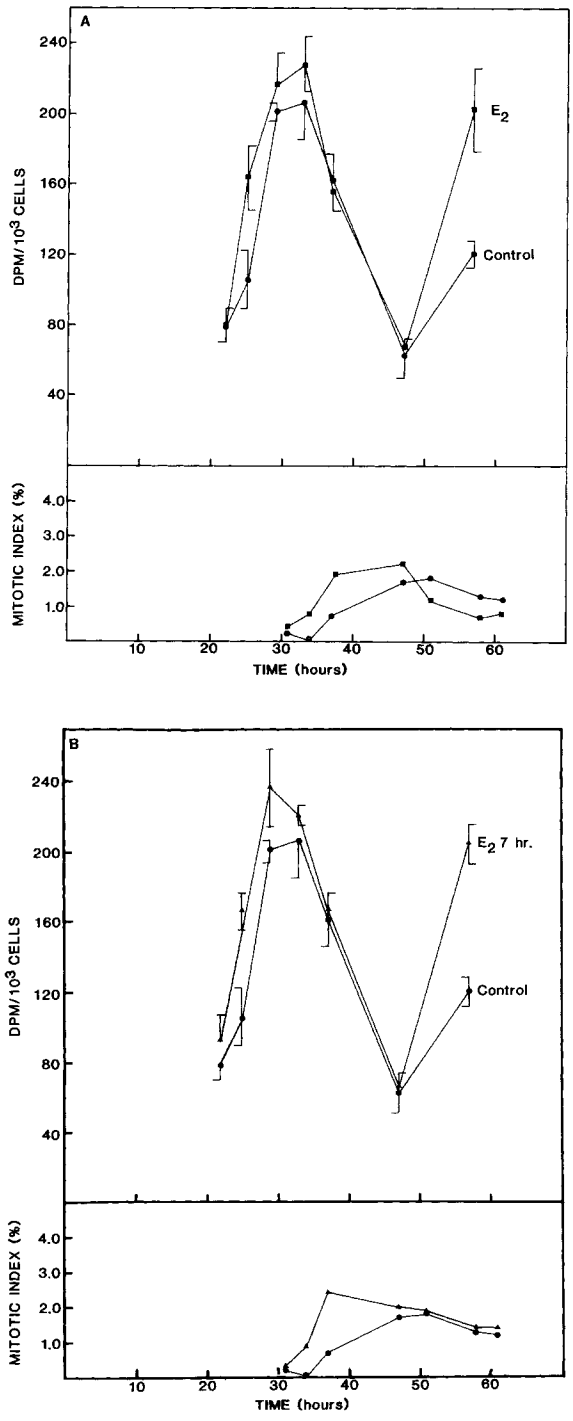


Fig. 6. Uptake of ^{3}H-dThd and mitotic index of cells with E_2 added at various times following Percoll enrichment. **(A)** E_2 added 2 hr after selection (■). **(B)** E_2 added 7 hr (▲) after selection. Control without E_2 (●). Data for both A and B were generated concurrently. Each point is the mean ± SD of triplicates. At 25 hr, E_2 stimulation of ^{3}H-dThd in both A and B is statistically significant ($P < .01$).

Similar experiments to those described in Figures 1–6 were conducted with the use of the CAMA-1R subline; except for a difference in cell cycle time, the effects of E_2 on the different cell phases were similar to results with CAMA-1N subline (results not shown).

Taking these experiments collectively, it appears that the early G1 phase is the most, and probably the only, E_2-sensitive period of the CAMA-1 cell cycle. Other cell phases may be refractory to E_2. The presence of E_2 during the S to early G2 period appears to be inhibitory. The possibility of G2/M cells responding to E2 requires further experimentation.

DISCUSSION

Variability of the cell cycle has been explained by the constant transition probability model, which states that when cells initiate the cell cycle they are essentially identical and that most of the variability in transit times occurs in the G1 phase [16]. During this phase, each cell undergoes a single random process with constant probability per unit time that takes it into the S phase. Results from this study and a previous report [4] that indicate that E_2 shortens G1 duration and increases the G1/S transition correspond well with this model; that is, E_2 alters the probability of cells entering into DNA synthesis without an apparent effect on the rates of other periods of the cycle.

Estrogen-stimulated mammary tumor growth has been studied quite extensively in the last two decades. However, there is a paucity of precise information regarding the mode of E_2 action on cell proliferation and its influence on cell cycle kinetics. Most of the studies—for example, the finding that physiological levels of E_2 shorten the cell generation time primarily at the G1 phase of the cell cycle in mature mouse uterine cells [7] and that E_2 enhances the rate of MCF-7 cell proliferation with an accumulation of S population of cells [8]—have employed nonsynchronized populations. Tamoxifen, a nonsteroidal anti-estrogen, blocks G1 phase cells' progression in the MCF-7 cell line [9–11]. This action of tamoxifen is said to compete for a common E_2-regulated event in promoting G1 exit [17]. Similar findings were seen with T47D [18] and CAMA-1 cells [2,3]. While these studies are important, there is a need to determine the precise period in the G1 phase in which E_2 exerts its influence. Such studies can best be achieved with synchronized populations. In this study and in a previous study [4], we have successfully demonstrated that the three methods of cell synchronization used for CAMA-1 cells, namely, dThd block at G1/S boundary, nocodazole arrest at G2/M boundary, and serum deprivation at early G1 phase [4], are of particular value in monitoring E_2 effects on the cell cycle kinetics and in testing the sensitivity of the different cell phases to estrogen stimulation or inhibition. Our result shows that the cell-proliferating property of E_2 lies mainly in promoting G1 to S phase transition, ie, shortening of the G1 duration, and causes more and faster turnover of E_2-stimulated cells per cell cycle. The early G1 phase, possibly G2/M, is sensitive to E_2 stimulation, whereas other periods are either refractory to or inhibited by E_2. These findings are further strengthened by observations showing that anti-estrogens such as tamoxifen or nafoxidine inhibit progression of cells at the G1 phase and prolong the generation time [19]. Results herein will aid the design of future experiments to identify the estrogen-regulated events that modulate G1 exit.

Our present understanding of the mechanism of estrogen action relating to cell proliferation is very inadequate. In order to elucidate this mechanism, the period of the cell cycle in which E_2 acts must be determined precisely so that estrogen-regulated events during this period can be identified and investigated in respect to their relation to estrogen-induced cell proliferation. In this report, we demonstrated that the very early G1 phase, and probably G2/M, is sensitive to E_2 stimulation. Estrogen-induced cell proliferation cannot take place without first allowing the cells at this stage to be exposed to E_2. This finding confirms our previous report that, when CAMA-1 cells were arrested at the early G1 phase by serum deprivation [4], E_2 elicited a stimulatory effort to early G1 phase cells, resulting in promoting more and faster exit of these cells to S phase. During serum deprivation, synchronized cells had a lengthy G1 phase. Without estrogen treatment, a mean G1 duration lasted for 38 hr and 52 hr for CAMA-1R and CAMA-1N sublines, respectively, and E_2 shortened the mean G1 duration by as much as 24 hr for both sublines. Of particular importance is the fact that these cells fail to respond to estrogen stimulation in the absence of serum. In other mammalian cells, the G1 phase has been documented to be affected by external growth conditions such as serum concentration, nutrients, cell density, and growth factors [20,21]. For example, in the mouse embryonic BALB/C 3T3 cell line [22], some growth factors, such as platelet-derived growth factor, are responsible for inducing arrested G0 cells to a state of competence so that they will respond to a second set of growth factors, termed progression factors. Among them are epidermal growth factor, which promotes the competent cell to progress to the G0/G1 boundary, and somatomedin, which promotes these cells to traverse the G1 phase. Estrogen-induced synthesis of growth factors in target cells has been well documented [23]; however, the role of these factors in estrogen-induced tumor growth remains obscure. The need for advancing our understanding in areas such as cell proliferation in relation to these growth factors, whether E_2-induced or not, their temporal effects at the M/G1 period of the cell cycle, and the possible mediating or synergising effects of these growth factors for estrogen-regulated events at the M/G1 period, has become apparent both for their fundamental importance in explaining E_2-induced tumor growth as well as for potential clinical applications.

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