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Progestins Both Stimulate and Inhibit Breast Cancer Cell Cycle Progression while Increasing Expression of Transforming Growth Factor α, Epidermal Growth Factor Receptor, c-*fos*, and c-*myc* Genes

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This study documents a biphasic change in the rate of cell cycle progression and proliferation of T-47D human breast cancer cells treated with synthetic progestins, consisting of an initial transient acceleration in transit through G_1, followed by cell cycle arrest and growth inhibition. Both components of the response were mediated via the progesterone receptor. The data are consistent with a model in which the action of progestins is to accelerate cells already progressing through G_1, which are then arrested early in G_1 after completing a round of replication, as are cells initially in other phases of the cell cycle. Such acceleration implies that progestins act on genes or gene products which are rate limiting for cell cycle progression. Increased production of epidermal growth factor and transforming growth factor α, putative autocrine growth factors in breast cancer cells, does not appear to account for the initial response to progestins, since although the mRNA abundance for these growth factors is rapidly induced by progestins, cells treated with epidermal growth factor or transforming growth factor α did not enter S phase until 5 to 6 h later than those stimulated by progestin. The proto-oncogenes c-*fos* and c-*myc* were rapidly but transiently induced by progestin treatment, paralleling the well-known response of these genes to mitogenic signals in other cell types. The progestin antagonist RU 486 inhibited progestin regulation of both cell cycle progression and c-*myc* expression, suggesting that this proto-oncogene may participate in growth modulation by progestins.

The precise nature of the cellular response to the steroid hormone progesterone in vivo is species specific and varies with cell type and with stage of development, even within particular target organs. The mitotic activity of human mammary epithelium peaks in the later, secretory, stage of the menstrual cycle, when serum concentrations of both estrogen and progesterone rise (44), although it has not been demonstrated that these hormones directly stimulate such proliferation. Milk products are synthesized and secreted during lactation, by cells lining a system of branched ducts terminating in alveoli, which develops during pregnancy from the simpler structures formed during adolescence. The role of progesterone in this process is to stimulate proliferation of the glandular epithelium, i.e., to promote branching of the ducts and lobuloalveolar development (67). Thus, although the regulation of breast epithelial cell proliferation is not well understood, particularly in the human, progesterone appears to be a major stimulus for this tissue during the menstrual cycle and in pregnancy (reviewed in reference 13), in contrast with the marked inhibition of estrogen-induced proliferation and induction of differentiation by progesterone in uterine tissues (13).

Progestin inhibition of breast cancer cell growth in tissue culture has been well documented in publications from a number of laboratories (32, 33, 63, 66, 70) and is the result of a decrease in the rate of entry into S phase (66). Early suggestions that only estrogen-induced proliferation could be inhibited have not generally been supported by more recent data, including the failure of estrogen addition to diminish the effects of progestins (66), and the demonstration, in this and other reports (63), of growth inhibition in cultures depleted of estrogen. Synthetic progestins are an effective treatment for up to a third of breast cancer patients, but the mechanisms for this antitumor activity are unclear since receptor status has not always been found to correlate with response (references 13 and 62 and references therein). Although, consistent with the effects of progesterone in vivo, progestin stimulation of rodent mammary cells in organ or cell culture has been repeatedly observed, for normal human breast cells or cell lines the data are relatively sparse and less consistent (reference 13 and references therein) and in established breast cancer cell lines stimulatory responses, evidenced by increases in cell number, have only recently been recorded (8, 30).

Although the molecular basis of steroidal regulation of gene transcription has been well documented (4), the mechanisms linking steroid hormone action with cell cycle control in hormone-responsive cells have not been delineated. Much recent evidence supports the view that peptide growth factor pathways are intimately involved in the proliferative response of breast cancer cells (2, 17), but it is not yet clear whether modulation of growth factor production and/or of receptor numbers has a causative role in the control of breast cancer cell proliferation. Furthermore, there are only limited cell cycle kinetic data to provide a basis for understanding the mechanisms underlying such hormone-dependent proliferation. One way to address such questions is the use of defined culture conditions to facilitate the dissection of these complex pathways of growth control. Therefore, this study focuses on the effects of synthetic progestins on the proliferation and cell cycle progression of breast cancer cells in serum-free, estrogen-depleted medium and examines the

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role of progestin regulation of growth-related genes. In this model system, the substantial but submaximal proliferation rates achieved allow for the detection of either stimulation or inhibition of cell cycle progression.

MATERIALS AND METHODS

Reagents. Steroids, prepared as 1,000- or 2,000-fold concentrated stocks in absolute ethanol and stored at −20°C, were obtained from the following sources: ORG 2058 (16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione), Amersham Australia; R5020 (17α-21-dimethyl-19-norpregn-4,9-diene-3,20-dione), Du Pont (Australia) Ltd; RU 486 [17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one], J.-P. Raynaud of Roussel-Uclaf, France; medroxyprogesterone acetate (MPA; 17α-acetoxy-6α-methyl-4-pregn-4-ene-3,20-dione), Dudley Jacobs of Upjohn Pty Ltd, Sydney, Australia; and dexamethasone (9-fluoro-11,17,21-trihydroxy-16-methylpregn-1,4-diene-3,20-dione), Sigma Chemical Co., St. Louis, Mo. ICRF 159 (Razoxane) was supplied by ICI Pharmaceuticals Division, Macclesfield, Cheshire, United Kingdom, and stored and administered as previously described (66). Tissue culture reagents were purchased from standard sources. Human recombinant transforming growth factor α (TGFα; Bachem Feinchemikalien AG, Bubendorf, Switzerland) and murine epidermal growth factor (EGF; Collaborative Research, Bedford, Mass.) were dissolved in distilled water, stored in aliquots at −20°C, and used without filtration.

Cell culture. Stock cultures of T-47D and MCF-7 cells were maintained as previously described but without antibiotics in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (66). The medium used for experiments consisted of RPMI 1640 containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 20 mM), sodium bicarbonate (14 mM), L-glutamine (6 mM), and gentamicin (20 μg/ml). It was without phenol red, except as noted below, since some preparations of this pH indicator have estrogenic activity (6). Serum-free medium (SF medium) additionally contained 300 nM human transferrin; alternately, the base medium was supplemented with filter-sterilized charcoal-stripped FCS (SFCS) (59).

Cell kinetic studies. To deplete the cells of any sequestered steroids in particular estrogens, prior to each experiment, cells taken from stock cultures were passaged for 5 to 7 days in medium containing 10% SFCS, with two changes of medium at 1- to 3-day intervals. These steroid-depleted cells were replated into replicate flasks (1×10^5 or 1.5×10^5 cells per 25-cm^2 flask) in 5 ml of medium containing 15% SFCS. On 2 successive days thereafter, the medium was replaced, first with SF medium and then with SF medium containing 1 μg/ml (MCF-7) or 10 μg/ml (T-47D) porcine or human insulin (CSL-Novo, Sydney, Australia). This medium was not further replaced during the course of the experiment. Preliminary experiments established that in SF medium without insulin these cell lines grew slowly, with doubling times typically 4 to 6 days or more, while in SF medium containing 1 to 10 μg of insulin per ml the growth rate was comparable with (but consistently slower than) that achieved in the presence of 10% FCS in parallel flasks. In the experiments presented here, insulin-stimulated cell growth in SF medium was exponential, with a typical population doubling time of 2.0 to 2.5 days. Stock cultures in serum-containing medium had doubling times of approximately 1.5 to 2.0 days.

Each experiment commenced 2 to 4 days after the change to insulin-containing SF medium, with the addition of steroid or growth factor directly into the medium. At the indicated times thereafter, flasks were harvested with 0.05% trypsin–0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl); the cells were resuspended in tissue culture medium and finally, after cell counting using a hemacytometer under phase-contrast microscopy where appropriate, stained for later DNA analysis by the addition of ethidium bromide (40 μg/ml) and mithramycin (12.5 μg/ml) in the presence of 0.2% (vol/vol) Triton X-100 as previously described (55). DNA histograms were obtained by using a FACStar flow cytometer (Becton Dickinson Immunocytochemistry Systems, Mountain View, Calif.), and the cell cycle phase distribution was estimated by using the manufacturer's DNA analysis software (SFIT). Each histogram contained 30,000 events and typically had a coefficient of variation for the G_1 peak of 2 to 3%.

RNA preparation and Northern (RNA) analysis. T-47D cells from stock cultures were plated into 150-cm^2 flasks in phenol red-containing medium with 5% FCS, and 4 days later, during exponential growth, the cells were changed to medium containing phenol red and 1% SFCS. Treatment with 1 to 10 nM ORG 2058 commenced 24 h later. At each time point, RNA was extracted from duplicate flasks by using guanidinium isothiocyanate-cesium chloride as previously described (1). Alternately, RNA was extracted from duplicate or triplicate 150-cm^2 flasks of cells set up in phenol red-free medium with 15% SFCS and on successive days changed to SF medium and then SF medium containing insulin, in the same fashion as used for the cell kinetic studies.

A 20-μg aliquot of total cellular RNA was used for Northern analysis by standard techniques (1). Briefly, the denatured RNA was electrophoresed through a 1% agarose–2.2 M formaldehyde gel and capillary transferred to a Zetaprobe nylon membrane after partial alkaline hydrolysis. This membrane was later hybridized to cDNA probes labeled with [α-^{32}P]dCTP by nick translation or random priming to a specific activity of 3×10^8 to 10×10^8 dpm/μg. Excess probe was removed by washing the membrane at a maximum stringency of 0.2× SSC–1% sodium dodecyl sulfate (SDS) at 65°C (1× SSC contains 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). After autoradiography using Kodak X-Omat film, relative mRNA abundance was quantitated by densitometric analysis using a Bio-Rad model 620 video densitometer and the Bio-Rad 1-D Analyst software. Reproducibility of RNA loading was estimated by densitometric analysis of autoradiographs from filters reprobed with a [γ-^{32}P]ATP-end-labelled synthetic 30-nucleotide probe complementary to the 18S rRNA subunit (12). Excess oligonucleotide was removed by washes with a highest stringency of 2× SSC–1% SDS (65°C).

Proto-oncogene mRNA expression was measured by using as probes a 0.45-kb *Pst*I exon 2 fragment of the human c-*myc* gene (43), provided by Geoff Symonds, The Children's Medical Research Foundation, Sydney, Australia, and a 3.1-kb fragment of the coding region of human c-*fos* (Amprobe, Amersham). The 1.35-kb human TGFα cDNA (15) encompasses the entire coding region for the 160-amino-acid precursor of TGFα; the 2.0-kb human EGF cDNA, phEGF-15 (5), is within the coding region of the 1,207-amino-acid EGF precursor. The 1.8-kb human EGF receptor cDNA clone encodes the binding domain and part of the transmembrane domain (46).

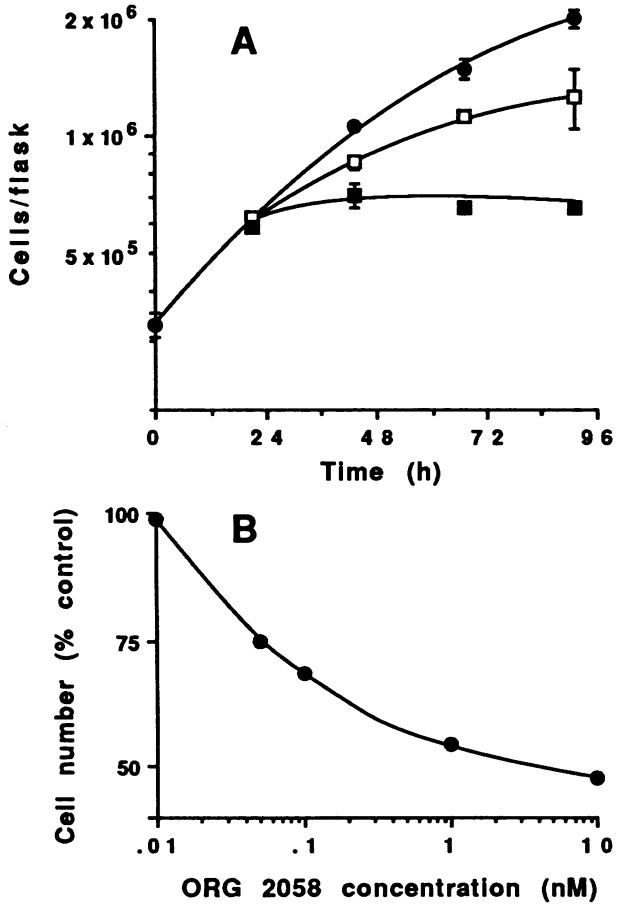

FIG. 1. Growth inhibition by ORG 2058 in SF medium. T-47D cells seeded into 25-cm^2 flasks were treated with the synthetic progestin ORG 2058 2 to 4 days after growth stimulation by insulin in SF medium; the cells were then harvested by trypsinization, and cell numbers were determined. (A) Points represent the means and standard errors of triplicate counts in a representative experiment for cells treated with vehicle (control; ●) or ORG 2058 (□, 0.1 nM; ■, 10 nM). (B) Cell numbers are expressed relative to those in control, vehicle-treated flasks after two population doublings of control cells. The data have been pooled from duplicate or triplicate determinations in seven separate experiments.

RESULTS

Effects of progestins on growth in SF medium. T-47D breast cancer cells were stimulated to proliferate by the addition of insulin (10 μg/ml) to SF medium in estrogen-depleted conditions. Treatment with the synthetic progestin ORG 2058 resulted in a concentration-dependent decrease in the rate of proliferation (Fig. 1A). The decrease in growth rate was not apparent until control cells had completed approximately one round of replication and doubled in number. Figure 1B shows the concentration dependence of cell number in ORG 2058-treated cultures relative to those in control, vehicle-treated flasks after two doublings of the control cells. It is in close agreement with the concentration dependence of another progestin, MPA, for the same number of control population doublings in the presence of 5% serum; we have shown previously that MPA and ORG 2058 are approximately equipotent in inhibiting the proliferation of this cell line (66). Thus, the growth-inhibitory potency of progestins appears to be little affected by the absence of either serum or estrogen.

Studies of the changes in cell cycle phase distribution during the first 12 to 18 h of ORG 2058 treatment in SF medium revealed the presence of a progestin-stimulated, apparently synchronous, cohort of cells, readily apparent in DNA histograms (Fig. 2A). These cells entered S phase after 8 to 10 h of ORG 2058 treatment (Fig. 2C) and, as indicated by the transient increase in the G_2+M fraction 6 h later (Fig. 2D), completed S phase and mitosis. The progestin-stimulated cells increased the S-phase fraction by >60%, from 21% in control cultures to a maximum of 34% after 12 h of ORG 2058 treatment. There was a concomitant decrease in the percentage of cells in G_1 phase. T-47D cells growing at apparently maximal rates, with a population doubling time of 22 h, have a steady-state S-phase percentage of 26 (66). This allows calculation of an S-phase duration of 5.8 h, a figure in good agreement with the ~6-h transit through S phase observed here. After the passage of the stimulated cohort, entry into S phase from G_1 was markedly inhibited (e.g., at 33 h; Fig. 2A), and there was a sustained decrease in the proportion of cells in S phase, such that the number of cells in S phase fell to 11% at 24 h (Fig. 2C). This decrease was maintained until at least 96 h (not shown).

Further experiments using a range of concentrations between 0.01 and 10 nM established the concentration dependence of the effects of ORG 2058 on the percentage of cells in S phase. Figure 3 shows data from times representative of the initial increase (11 to 14 h) and the subsequent decrease (24 to 36 h) in the S-phase percentage. For both components of the response, concentrations of 0.01 nM were ineffective, whereas concentrations of 0.1 nM or above were maximally effective. Growth inhibition, in contrast, increased gradually with concentration above 0.1 nM (Fig. 1), most likely reflecting a concentration-dependent resumption of cell proliferation despite continued progestin exposure (66).

MCF-7 cells were also treated with ORG 2058 in a similar procedure to determine whether the biphasic effects of this progestin were restricted to T-47D cells. Although this cell line is much less sensitive than T-47D, in a study of five receptor-positive cell lines, it was one of only two other than T-47D showing significant growth inhibition at progestin concentrations of ≤10 nM (66). The changes in cell cycle phase distribution of MCF-7 cells treated with 10 or 100 nM ORG 2058 in SF medium supplemented with insulin at 1 μg/ml were small but showed a transient increase and later decrease in the proportion of cells in S phase (Fig. 4), paralleled by changes in the proportion of cells in G_1 phase. These effects did not become more pronounced with further treatment (up to 3 days, not shown) and were similar in time course to the changes in T-47D cell cycle phase distribution after ORG 2058 treatment. Thus, the biphasic response to progestins may be characteristic of a general response of progestin-sensitive breast cancer cells.

In culture conditions in which the percentage of control T-47D cultures in S phase was less than that achieved in insulin-containing SF medium (i.e., in unsupplemented SF medium or in medium containing SFCS but without insulin), both the initial increase and later decrease in the percentage of cells in S phase after ORG 2058 treatment were of reduced magnitude (Table 1) but still readily apparent. These data suggest that the magnitude of these changes in the percentage of T-47D cells in S phase is related to the proportion of proliferating cells and not to the specific culture conditions. Therefore, stimulation of cell cycle progression by progestins does not result from entry of nonproliferating cells into

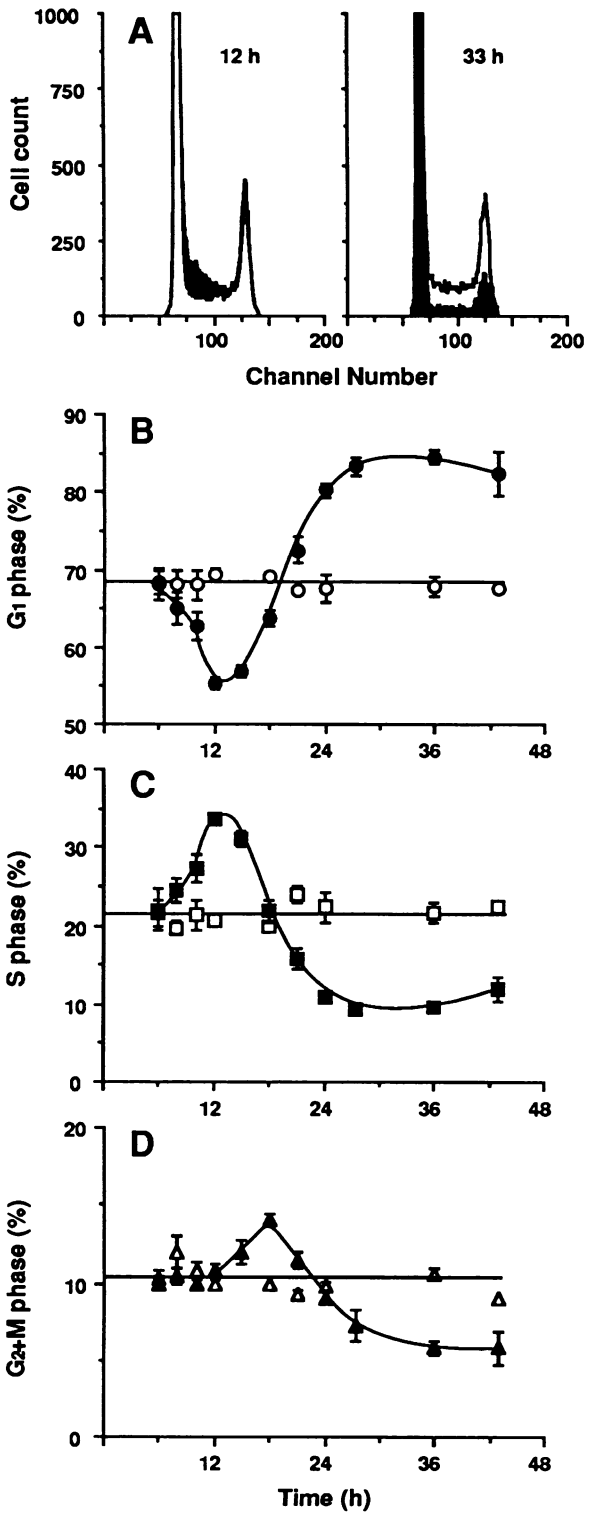

FIG. 2. Effect of ORG 2058 on cell cycle phase distribution. T-47D cells treated with the progestin ORG 2058 while growing exponentially in insulin-containing SF medium were harvested by trypsinization and stained for DNA content analysis by flow cytometry. Cell cycle phase distributions were calculated by computer fitting of the resulting histograms. (A) Superimposed histograms obtained from control (open area) and treated (1 nM ORG 2058; solid area) populations. An increase in the number of cells in the early part of S phase after 12 h of treatment with ORG 2058 is apparent, while at 33 h there is a marked decrease in S- and G_2+M-phase cells. (B to D) Time course of changes in the proportion of cells in G_0/G_1 (○, ●), S (□, ■), or G_2+M (△, ▲) phase upon treatment with vehicle (open symbols) or ORG 2058 (0.1 to 10 nM; closed symbols). These concentrations of ORG 2058 were shown to be equally effective, and the data have therefore been pooled; points represent means ± standard errors of data from six separate experiments.

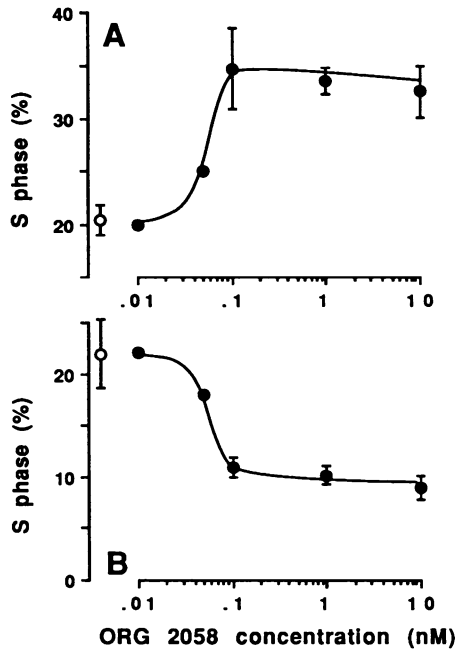

FIG. 3. Concentration dependence of effect of ORG 2058 on percentage of cells in S phase. T-47D cells treated with the progestin ORG 2058 at the indicated concentrations while growing exponentially in insulin-containing SF medium were harvested by trypsinization and stained for DNA content analysis by flow cytometry. Cell cycle phase distributions were calculated by computer fitting of the resulting histograms. Shown are means ± standard errors of data from cells harvested after exposure times representative of the initial increase (11 to 14 h; A) and later decrease (24 to 36 h; B) in S-phase percentage in six separate experiments. Open symbols, control; solid symbols, ORG 2058 treated.

the cell cycle but rather results from an action on cells already in G_1 phase. The time course of the subsequent decrease in S-phase percentage was only slightly slower in serum-free, estrogen-depleted conditions than in the presence of serum, despite the increase in doubling time from ≤1 day to ≥2 days. The doubling in cell numbers before any change in growth rate, noted above, implies that almost all cells could complete the cell cycle and divide once in the presence of the drug and thus confirms our previous conclusion of an early G_1 site of progestin inhibition (66). However, it appears that much of the effective lengthening of G_1 at slower growth rates occurs before the point at which progestins arrest cell cycle progression.

The hypothesis that the initial action of progestins was to transiently increase the rate at which cells entered S phase was tested in a stathmokinetic experiment. Pretreatment of T-47D cells with ORG 2058 for 6 h was followed by addition of ICRF 159 at 50 μg/ml, a nontoxic concentration of this inhibitor of cytokinesis. The effect of ICRF 159 is to prevent

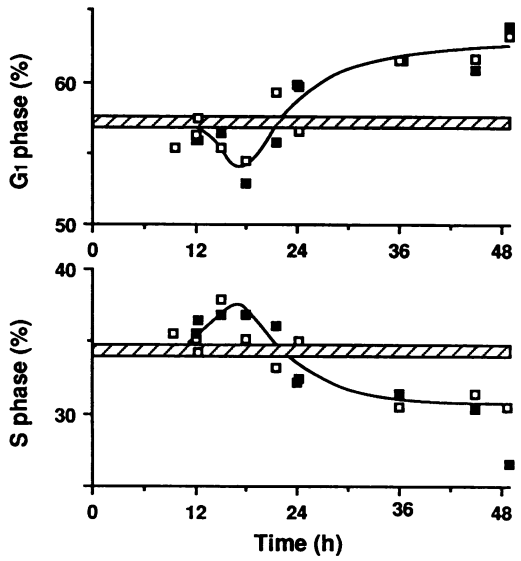

FIG. 4. Changes in MCF-7 cell cycle phase distribution after ORG 2058 treatment. MCF-7 cells proliferating in SF medium containing insulin (1 μg/ml) were treated with 10 (□) or 100 (■) nM ORG 2058 and harvested for DNA analysis by flow cytometry at intervals. Data shown are the results of computer fitting of the resulting histograms. Hatched bar shows mean ± 1 standard deviation of 11 control samples harvested throughout the experiment.

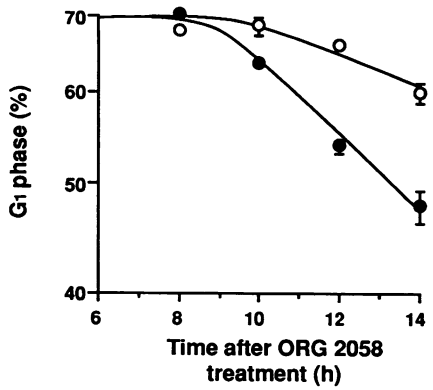

FIG. 5. Effect of pretreatment with ORG 2058 on the rate of exit from G_1 phase. After 6 h of pretreatment with the progestin ORG 2058 (0.1 or 10 nM), T-47D cells growing exponentially in insulin-containing SF medium were treated with ICRF 159 to inhibit cell division. Replicate flasks were harvested at various times thereafter, the cells were stained for DNA content analysis by flow cytometry, and the proportion of cells in G_1 phase was determined. Data from cells treated with 0.1 or 10 nM ORG 2058 were not significantly different and have been pooled. Points represent means ± ranges of duplicate (control; ○) or means ± standard errors of quadruplicate (ORG 2058 treated; ●) histograms from separate flasks.

cells completing mitosis and entering G_1; therefore, any decrease in the percentage of cells in G_1 phase in its presence reflects the transit of cells into S phase. Figure 5 shows that between 10 and 14 h after commencement of ORG 2058 treatment, the rate of exit from G_1 phase was significantly increased ($P = 0.001$; analysis of variance for interaction between time and treatment) approximately twofold compared with control cells. This is the same time frame as observed for increases in S phase, which were of the same magnitude in either the presence or absence of ICRF 159. The half-time for residence in G_1 was estimated from linear regression of time (10 to 14 h) against log G_1-phase percentage. It decreased from 20 h for control cells (95% confidence interval: 15 to 30 h) to 11 h for treated cells (95% confidence interval: 9 to 13 h).

TABLE 1. Effect of ORG 2058 on slowly proliferating T-47D cells[a]

Culture condition	ORG 2058 concn (nM)	% of cells in S phase		
		Control (mean ± SEM)	Maximum (15–18 h)	Minimum (33–44 h)
SF medium	Control	10.1 ± 0.5 (n = 4)		
	1		15	6.5
	10		14	6.0
1% SFCS	Control	17.0 ± 0.4 (n = 10)		
	0.1		29	12
	1		24	9
	10		26	9

[a] T-47D cells were treated with ORG 2058 at the indicated concentrations in phenol red-free, insulin-free culture conditions in which the proliferation rate was slower than in insulin-supplemented SF medium. S-phase fractions were measured by DNA flow cytometry. Data from control cultures harvested over the duration of the experiment have been pooled.

To establish that the biphasic changes in the percentage of cells in S phase were also characteristic of progestins other than ORG 2058, T-47D cells were treated in parallel with the synthetic progestins ORG 2058, R5020, and MPA at 1 nM. The progestins all caused biphasic changes in S-phase percentage of similar magnitude, increasing this value after 12 h exposure and later (33 h) decreasing it; reductions in S-phase percentage were reflected in decreases in cell number relative to the control (Fig. 6). The glucocorticoid dexamethasone (100 nM) had no effect on either cell number or cell cycle phase distribution (Fig. 6), confirming that neither part of the response to ORG 2058 was mediated via binding to the glucocorticoid receptor. Cells were also treated with RU 486, which binds to both the glucocorticoid and progesterone receptors but acts primarily as a progestin antagonist in breast cancer cells (3, 31). There was no evidence for an initial transient increase in the S-phase percentage upon treatment with 10 nM RU 486; indeed, some decrease was apparent, with a corresponding decrease in the relative cell number (Fig. 6), in agreement with other reports of inhibition of T-47D proliferation by RU 486 (3, 24, 31).

Antiprogestin antagonism of the progestin response. Since the initial increase in S-phase percentage was apparent upon treatment with several progestins but not the antiprogestin RU 486, we investigated whether RU 486 might antagonize this effect. Both the transient increase and later decrease in S-phase percentage resulting from treatment with 0.1 nM ORG 2058 were antagonized by RU 486 in a concentration-dependent fashion, with 50% inhibition of the ORG 2058-induced effects occurring at RU 486 concentrations of between 0.05 and 0.1 nM. Further experiments were designed in an attempt to separate the two components of the T-47D response to progestin treatment. Cells were exposed to 0.1 nM ORG 2058 together with 0.1 nM RU 486, added 1 to 9 h after ORG 2058. Addition of RU 486 3 h or less after exposure to ORG 2058 substantially reduced not only the initial increase but also the subsequent decrease in the

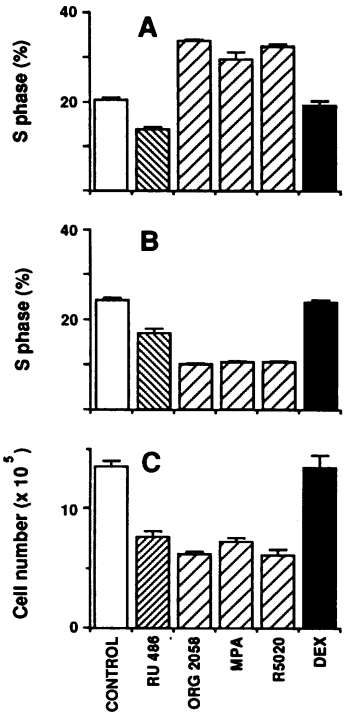

FIG. 6. Effects of RU 486, progestins, and dexamethasone on T-47D cells. T-47D cells proliferating in SF medium supplemented with insulin (10 μg/ml) were treated with either the progestin antagonist RU 486 (10 nM), the progestins ORG 2058 (1 nM), MPA (1 nM), and R5020 (1 nM), or the glucocorticoid dexamethasone (DEX; 100 nM) and subsequently harvested for DNA analysis or cell counts. The means and ranges of duplicate histograms at times representative of the initial increase (12 h; A) and later decrease (33 h; B) in the percentage of cells in S phase are shown. Cell counts (C) are the means ± standard errors of triplicate determinations after 3 days of treatment, approximately two doubling times for control cells.

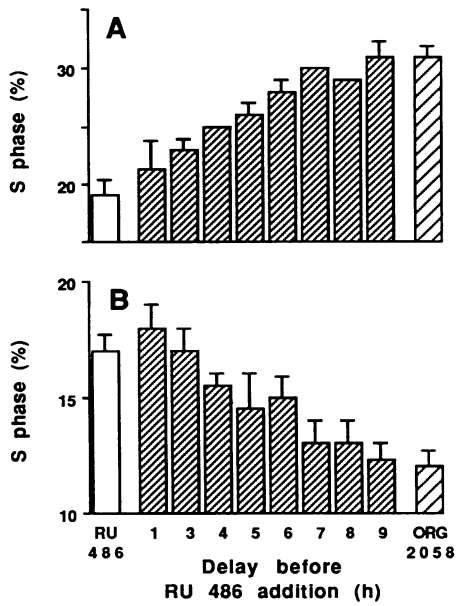

FIG. 7. Effect of delayed addition of RU 486 to T-47D cells treated with ORG 2058. Cells growing exponentially in insulin-containing SF medium were treated with the progestin ORG 2058 (0.1 nM) or vehicle. Additional treatment with RU 486 (0.1 nM) began 1 to 9 h later. The proportion of cells in S phase 12 h (A) and 27 h (B) after commencement of ORG 2058 treatment is shown for cells treated either with ORG 2058 or RU 486 alone or with both ORG 2058 and RU 486. Bars represent means ± ranges or standard errors of 2 to 4 histograms from two experiments except bars for RU 486 alone, which represent means ± standard errors of up to 10 histograms from cells treated for 0 to 12 h (A) or 15 to 27 h (B).

S-phase proportion of cells treated with ORG 2058 alone (Fig. 7). Addition of RU 486 after a delay of ≥7 h reduced the effect of ORG 2058 only slightly. In some experiments, decreases in S-phase percentage after treatment with 0.1 nM RU 486 alone were observed but affected the magnitude rather than the timing of the result. These data imply that in the majority of cells, the cell cycle kinetic changes require the presence of ORG 2058 for 3 h or more, in agreement with our previous postulate of an average delay of 4 to 5 h or more before progestin action on cellular replication, as determined from cell kinetic considerations (66). By 7 h, however, elaboration of progestin effects has become independent of progesterone receptor-mediated processes in essentially all of the sensitive population. Since on average the cohort of cells stimulated by progestins reaches S phase after 10 to 11 h of exposure, the progestin-accelerated event is likely to take place at least 5 h before entry into S phase. These estimates do not allow time for exchange of RU 486 for progesterone receptor-bound ORG 2058 and therefore represent the minimum exposure required.

Progestin regulation of growth factor mRNA. In many instances, changes in the rate of proliferation of breast cancer cells are accompanied by modulation of receptor numbers and/or production of autocrine growth factors; furthermore, the proliferation rate can be increased by the same growth factors (2, 17). On the basis of such evidence, it has been suggested that these changes might mediate the estrogen control of replication in breast cancer cells (17). It is thus possible that progestin regulation of genes for growth factors or their receptors could provide a mechanism by which progestins might increase the rate of proliferation. To test this possibility, the time course of changes in the expression of mRNA for TGFα, EGF, and the EGF receptor, through which both peptides exert their biological effects, was measured in T-47D cells treated with 10 nM ORG 2058. Northern analysis of total cellular RNA demonstrated a single hybridizable mRNA species for each growth factor, which rapidly increased in abundance upon ORG 2058 treatment (Fig. 8). Densitometric analysis showed that both TGFα and EGF mRNA levels were increased more than twofold within 3 h of ORG 2058 treatment (Fig. 8). The EGF mRNA remained elevated by more than fourfold between 3 and 24 h, while the TGFα mRNA reached a maximum, approximately threefold, increase at 6 h and returned to near control levels by 24 h. EGF receptor mRNA was detected as two hybridizable species. Its abundance was also elevated twofold within 3 h but did not reach a maximum until 12 h, a somewhat slower time course than observed for the growth factors. Although some recovery toward control levels was observed, the increase in EGF receptor mRNA abundance was maintained threefold above the control value for at least 24 h.

Since these data were compatible with rapid changes in growth factor production or sensitivity mediating the initial stimulation of proliferation by progestins, we next measured

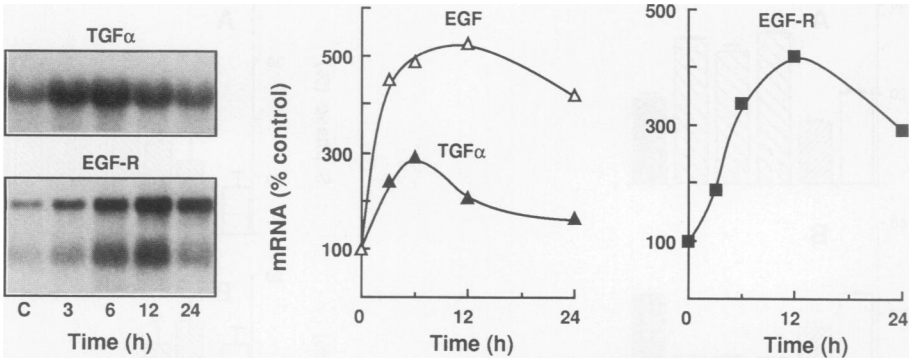

FIG. 8. Effects of 10 nM ORG 2058 on expression of mRNAs for TGFα, EGF, and the EGF receptor. Cells growing in the presence of 1% SFCS were treated with vehicle (C) or ORG 2058 (10 nM) and at the indicated times, duplicate 150-cm^2 flasks were harvested, and total cellular RNA was extracted for Northern analysis by standard procedures. The same filter has been probed successively for each mRNA species; the two mRNA species commonly observed for the EGF receptor are apparent. RNA loading was compared by reprobing the filter with a ^{32}P-end-labelled oligonucleotide probe complementary to the 18S rRNA subunit and varied by less than 10%. Similar changes in TGFα mRNA abundance were also seen for cells proliferating in SF medium supplemented with insulin.

the changes in S-phase percentage after addition of 10 nM ORG 2058, 1 or 10 nM EGF, or 1 or 10 nM TGFα to insulin-stimulated cells growing in SF medium and compared these changes with those after addition of FCS. The effects of 5 nM estrogen were also examined to determine whether steroid-induced stimulation of proliferation was invariably followed by growth inhibition in this model system. Both peptide growth factors stimulated increased entry of cells into S phase, and DNA analysis by flow cytometry (not shown) revealed a semisynchronous cohort of cells moving through S phase, similar to that observed upon ORG 2058 treatment. Increases in S-phase percentage were first detectable 15 h after, and reached a maximum ~21 h after, TGFα or EGF treatment (Fig. 9A) but then declined by 24 h as the stimulated cohort entered G_2+M. This time course for transit of S phase was slightly longer than observed for cells stimulated by ORG 2058 (Fig. 9). FCS more closely reproduced the time course of effects of ORG 2058 on cell cycle progression, although the changes in cell cycle phase distribution were delayed by more than 6 h in comparison with ORG 2058 (Fig. 9). Furthermore, the complex mixture of factors contained in FCS was as effective as the single agent, ORG 2058: both increased the proportion of cells in S phase from ~20% to more than 30%. Estrogen also increased the proportion of cells in S phase, which reached a maximum at approximately 21 h (Fig. 9B), more closely approximating the time course of stimulation by serum or peptide growth factors than that of progestins. At later time points, up to 45 h, no changes in cell cycle phase distribution suggestive of inhibition were observed. Both TGFα and EGF were equally effective at 1 and 10 nM, suggesting saturation of the effect by 1 nM. However, EGF was somewhat less effective than TGFα, and neither growth factor induced increases in S-phase percentage comparable in magnitude with those induced by ORG 2058 or by FCS. Although increased receptor numbers in the presence of ORG 2058 (19, 54) may increase cellular sensitivity and therefore increase the maximum effect, the accelerated entry into S phase upon ORG 2058 treatment preceded any effect by either growth factor by ~6 h and therefore is unlikely to result from actions of these growth factors on cellular replication.

Progestin regulation of proto-oncogene mRNA. We next postulated that since the effects of progestins in vivo include both proliferation and differentiation, the cell cycle-related targets for progestin action might include the proto-oncogenes c-*myc* and c-*fos*, which are regulated in response to diverse mitogenic stimuli (28, 36, 38, 49) and differentiation-inducing agents (25, 39). Both c-*fos* and c-*myc* were present at low but detectable levels in control cells, and their expression was transiently increased by ORG 2058 (Fig. 10). c-*fos* mRNA expression reached a maximum at 30 min and returned to approximately control levels within 2 h. In six of nine experiments, after 30 min of treatment with ORG 2058, 50 to 330% increases in c-*fos* mRNA expression relative to that in time-matched, vehicle-treated control cultures were observed. In some time course experiments, transient enhancement of c-*fos* mRNA expression in vehicle-treated control cultures was observed, possibly accounting for the undetectable effect of ORG 2058 in the other experiments. c-*myc* mRNA expression was maximal (up to eightfold greater than for the control) after 1 to 2 h of ORG 2058 treatment and returned to near control values by 6 h (Fig. 10). The increase in mRNA expression was apparent within 15 min (not shown) and, in the same experiments as those in which c-*fos* mRNA expression was measured, reached an average approximately 3.5-fold increase relative to the control by 30 min. The increases in mRNA abundance for c-*fos* and c-*myc* thus follow a time course typical of the response of these proto-oncogenes to mitogens in many cell types (for example, see references 28 and 36) and similar to that resulting from estrogen stimulation of breast cancer cells (69).

If the enhanced expression of c-*myc* and c-*fos* is involved in the accelerated cell cycle progression induced by progestins, as suggested by these data, RU 486 would be predicted to antagonize the response as part of its antagonism of progestin effects on cell proliferation. Therefore, cells were treated with either ORG 2058, RU 486, or a combination of both agents. RU 486 alone did not increase the expression of either proto-oncogene at any time point examined (15 min to 2 h; not shown). Antagonism of ORG 2058 effects on c-*fos* mRNA expression could not be unequivocally demonstrated because of the relatively small magnitude of the increase in mRNA expression. However, c-*myc* induction by ORG 2058 was entirely abrogated by simultaneous treatment with RU 486 (Fig. 11) at each time point examined (15 min to 2 h). Together, these results suggest that growth regulation by

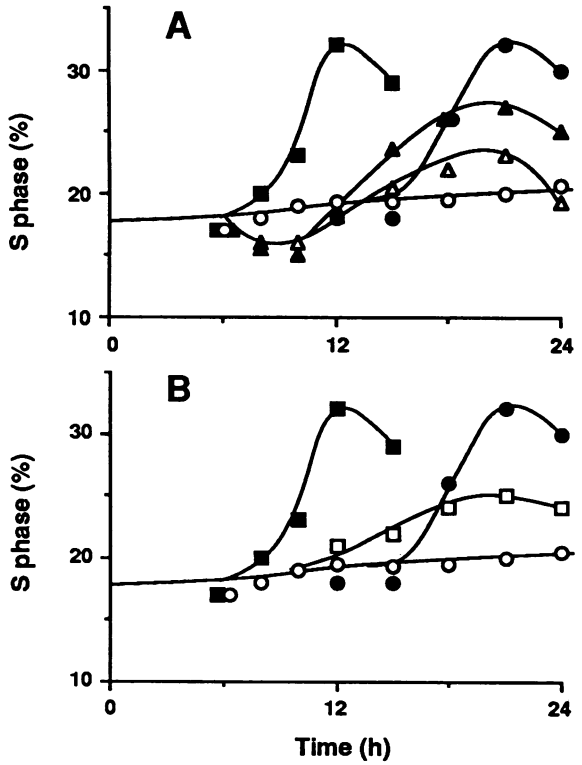

FIG. 9. Effects of serum, growth factors, estrogen, or ORG 2058 on proportion of cells in S phase. Replicate flasks of cells growing exponentially in insulin-containing SF medium were additionally treated with TGFα (1 or 10 nM; ▲) or EGF (1 or 10 nM, △) (A) or with 17β-estradiol (5 nM; □) (B). Results from flasks treated with FCS (final concentration, 10%; ●), ORG 2058 (10 nM; ■), or ethanol vehicle (○) are presented in both panels for clarity. At the indicated times, flasks of cells were harvested and stained for DNA content analysis by flow cytometry. EGF and TGFα were equally effective at 1 and 10 nM.

progestins may be paralleled by regulation of these proto-oncogenes, particularly c-*myc*.

DISCUSSION

We have shown in this report that a given concentration of progestin can stimulate as well as inhibit cell cycle progression, causing a biphasic change in the proportion of cells in S phase. This effect is mediated via the progesterone receptor, as determined from the following criteria: the maximal response occurred at low concentrations of ORG 2058; several synthetic progestins induced similar changes in cell cycle progression while under the same conditions the antiprogestin/antiglucocorticoid RU 486 or the glucocorticoid dexamethasone did not; and RU 486 inhibited the response in a concentration-dependent fashion. The maximal effects were seen at ORG 2058 concentrations of 0.1 nM, slightly lower than the affinity of ORG 2058 for the progesterone receptor in these cells (0.5 to 1.1 nM [58, 66]) and incompatible with the higher concentrations required for action via binding of progestins to the androgen or glucocorticoid receptors. The biphasic nature of the response to progestins distinguishes it from the effects of other agents which act via steroid receptors to regulate proliferation. For example, although antiestrogens and RU 486 inhibit proliferation in the same experimental design, this is not preceded by stimulation (unpublished data), and conversely, while estrogen stimulates the entry of cells into S phase, this is not followed by later inhibition. The initial progestin-stimulated increase in S-phase percentage was the result of a cohort of cells that entered S phase approximately 8 h after the commencement of ORG 2058 treatment and completed a round of replication at the normal rate. This cohort of cells had a half-time of residence in G_1 approximately half that of control, insulin-stimulated cells in SF medium and only slightly longer than that of rapidly cycling cells in the presence of serum (66). After the transient increase in S-phase percentage, progestin treatment of T-47D cells in estrogen-depleted conditions led to arrest in G_1 phase and

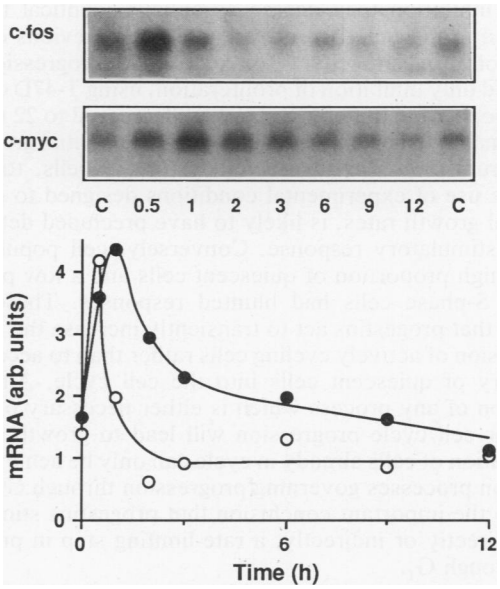

FIG. 10. Effect of ORG 2058 on expression of c-*fos* and c-*myc* mRNAs. Total cellular RNA was extracted for Northern analysis from triplicate 150-cm^2 flasks of cells growing in insulin-containing SF medium. A representative time course experiment of ORG 2058 (10 nM) treatment is shown. Points represent densitometric analysis of c-*fos* (○) and c-*myc* (●). Data at 0 h represent the mean of three vehicle-treated control samples harvested during the experiment. RNA loading was quantitated by reprobing the same filters with a ^{32}P-end-labelled oligonucleotide complementary to the 18S rRNA subunit and varied by <15%.

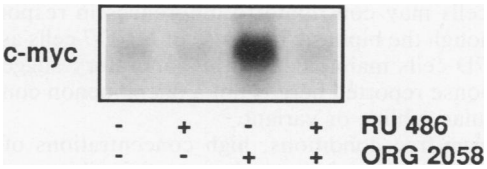

FIG. 11. Antagonism of ORG 2058 effect on expression of c-*myc* mRNA by RU 486. Total cellular RNA was extracted for Northern analysis from triplicate 150-cm^2 flasks of cells growing in insulin-containing SF medium. Cells were treated with ethanol vehicle, 100 nM RU 486, 10 nM ORG 2058, or 10 nM ORG 2058 added simultaneously with 100 nM RU 486 and harvested after 1 h. RNA loading was quantitated by reprobing the same filters with a ^{32}P-end-labelled oligonucleotide complementary to the 18S rRNA subunit and varied by <13%.

growth inhibition to a degree essentially identical to that observed in the presence of serum. In our previous examination of progestin effects on cell cycle progression we observed only inhibition of proliferation, using T-47D cells in which population doubling times had decreased to 22 to 24 h during maintenance in almost constant exponential growth in 10% serum (66). This characteristic of the cells, together with the use of experimental conditions designed to ensure maximal growth rates, is likely to have precluded detection of any stimulatory response. Conversely, cell populations with a high proportion of quiescent cells and a low proportion of S-phase cells had blunted responses. These data suggest that progestins act to transiently increase the rate of progression of actively cycling cells rather than to accelerate the entry of quiescent cells into the cell cycle. Although inhibition of any process which is either necessary or sufficient for cell cycle progression will lead to growth arrest, acceleration of cells already in cycle can only be achieved by action on processes governing progression through G_1. This leads to the important conclusion that progestins stimulate, either directly or indirectly, a rate-limiting step in progression through G_1.

Several strategies were used in attempts to define conditions under which the initial stimulation could be observed in isolation from the later growth inhibition. In T-47D cells, the two effects could not be separated on the basis of progestin concentration dependence or of concentration or time dependence of RU 486 antagonism: in each case, any reduction in magnitude of one component of the response was accompanied by a decrease in the magnitude of the other. Furthermore, in MCF-7 cells the degrees of both stimulation and inhibition were small. In part, this finding reflects progesterone receptor binding as the initial step in both responses, but it also implies a convergence of more distal actions. Long-term stimulation of proliferation by progestins was not observed in the experiments reported here and has not been observed in estrogen-depleted culture conditions by other authors (24, 63). Where progestin stimulation has previously been described, relative increases in cell number in progestin-treated cultures (30) are rarely more than twofold even after extended treatment, and in the same experimental design RU 486 concentrations of $\geq$100 nM lead to similar increases in cell number (7). These authors have recently reported increases in thymidine kinase activity upon progestin treatment: the stimulation is maximum after 1 day and declines toward control values thereafter (48). The apparently extremely low rates of growth achieved in the experiments of Moore et al. (48) may account for their ability to detect growth stimulation in isolation from inhibition but may also reflect other factors, including details of the experimental design. Alternately, variability among sublines of T-47D cells may contribute to differences in response (26, 58), although the biphasic response of MCF-7 cells as well as the T-47D cells maintained in this laboratory suggests that the response reported here is not a phenomenon confined to a particular subline or variant.

In serum-free conditions, high concentrations of insulin have been shown to abrogate the growth inhibitory effects of progestins on T-47D cells (63), although the magnitude of progestin-mediated growth inhibition in serum-containing medium was not altered by the presence of insulin in either that laboratory (63) or our own (37). One possible explanation of these results is that progestin-mediated growth inhibition may depend on the production of a cofactor, either already present in serum or rapidly induced by it, which remains below the critical concentration when SF medium is exchanged routinely (e.g., as in reference 63). A further possibility is that medium exchange accelerates the processes involved in the eventual recovery from progestin-mediated growth arrest (66).

On the basis of the data presented here, progestin action on cell cycle progression in T-47D cells can be interpreted as follows: during initial exposure, a rate-limiting process in mid-G_1 is accelerated, partially synchronizing a cohort of cells which then leaves G_1 ~5 h after completion of the rate-limiting process. After a delay during which necessary preparatory steps are completed, cells entering G_1 are arrested in early G_1; cells originally in S and G_2+M will continue through the cycle, contributing to the observed increase in cell numbers. These data thus provide experimental support for the essential features of a general model that we have proposed (13), which can account for many of the known effects of steroids, steroid antagonists, and growth factors on breast cancer cells and is compatible with the in vivo effects of progestins. Since in a number of tissues progestins can be viewed as differentiative, it was postulated as part of the model that growth arrest might be a consequence of the initiation of a differentiation program (13). The transient increase in cell cycle progression might then arise from a necessity for DNA replication before full expression of a differentiated phenotype after growth arrest, in turn accounting for the coordination in magnitude of the dual effects of progestins reported here. However, the present data do not exclude the possibility that progestin control of replication occurs by two independent mechanisms. If progestin-stimulated proliferation in vivo is of the same transient nature as that described here, increases in thymidine labelling or mitotic index of mammary tissue may not be indicative of sustained proliferation. Since long-term pharmacological use of progestins in combination oral contraceptives or hormone replacement therapy is becoming increasingly common and such treatment has been advocated as a means of reducing the risk of breast cancer (23), this issue requires more detailed examination.

To identify potential molecular mechanisms by which progestins interact with the signals governing breast cancer cell replication, we examined the role of progestin-induced changes in the production of EGF and TGFα, potential autocrine growth factors, and the EGF receptor, through which both act. Several lines of evidence implicate EGF and/or related peptides, including TGFα, not only in the overall control of proliferation in human breast cancer cells but specifically in the response to progestins. Increased binding of EGF to progestin-treated cells (54) is accompanied by enhanced expression of mRNA for the EGF receptor (19, 53). Several laboratories including our own have observed some degree of EGF reversal of progestin effects on cell proliferation (37, 50, 63), and EGF and TGFα mRNA levels are increased by progestins (51, 52). However, in the case of EGF at least, this was not accompanied by increased secretion of mature 6-kDa peptide, and biological activity of the high-molecular-weight secreted product was not demonstrated (52). Regulation of EGF or TGFα production is unlikely to explain the long-term inhibition of growth by progestins since EGF and TGFα mRNA levels remain elevated for >24 h at concentrations of ORG 2058 which are inhibitory within that time. In previous studies using poly(A)$^+$ RNA, TGFα mRNA induction was not detectable until 12 h (51). However, the earlier increase shown here is sufficiently rapid to be compatible with accelerated progress through G_1 resulting from increased production of these growth factors. We tested this idea by examining the time

course of changes in S phase upon growth factor treatment and found that neither TGFα nor EGF caused increases in the proportion of cells in S phase until more than 5 to 6 h after the progestin-induced effect. Furthermore, neither peptide induced increases in the proportion of cells in S phase of more than ~50% of that seen with ORG 2058. These results are not compatible with a causative role for EGF or TGFα in progestin-mediated regulation of breast cancer cell proliferation, nor do they support modulation of mRNA for these growth factors as a necessary result of changes in proliferation rate.

The limited number of progestin-regulated genes so far identified includes only one, c-*myc*, which might have a role in cell cycle control. Progesterone inhibits estrogen-induced mitosis in the chick oviduct, and this is accompanied by a rapid (within 5 to 10 min) down-regulation of c-*myc* mRNA (20), reminiscent of the down-regulation of this gene reported after treatment of a number of cell lines with differentiation agents (10, 25, 40, 72). A transient disappearance of Myc protein has been suggested as a molecular switch which in some cases can control the initiation of differentiation during G_1 (14, 22), perhaps at G_D (57). G_D, described by Scott et al. (65) on the basis of extensive studies of the differentiation of 3T3 preadipocytes in vitro, represents the point in early G_1 from which the alternative pathways of proliferation or differentiation diverge. There are parallels between growth arrest by progestins and the G_D state, including the kinetics of growth arrest within G_1 (65) as well as increased expression of differentiated features. In T-47D cells in tissue culture, there is evidence that progestins induce changes, not solely the results of growth arrest, which are suggestive of increases in functional differentiation. These responses include accumulation of fatty acid synthetase protein and mRNA (11) and of lipid, the latter the result of increased synthesis of triglyceride with medium-chain fatty acids (35), a major component of milk fat.

c-*myc* and c-*fos* are transiently induced by a range of polypeptide mitogens (28, 36, 38, 49) and can also be regulated by steroids: estrogen induces both proto-oncogenes, in target tissues in vivo (68, 71) and in breast cancer cell lines (18, 69, 73). While c-*fos* is induced by differentiative agents (25, 39, 47), c-*myc* is often down-regulated, as noted above, although there are reports of its induction during phorbol ester-induced differentiation of leukemic B cells (41) and nerve growth factor-induced differentiation of PC12 pheochromocytoma cells (27). Interestingly, in both of these cases of c-*myc* induction, differentiation does not necessarily exclude proliferation: normal B cells continue to proliferate during the early phase of maturation, while PC12 proliferation has been reported to be enhanced by nerve growth factor (29), perhaps as a result of a necessity to complete a cell cycle in order to reach the G_D state in G_1 (61). The induction of c-*myc* and c-*fos* upon progestin treatment of breast cancer cells may reflect an analogous response. The induction of c-*myc* at least appears to be related to changes in proliferation rate, since RU 486 antagonizes the effects of ORG 2058 on both cell cycle progression and c-*myc* expression. Progestin response elements within the promoter regions of either c-*myc* or c-*fos* have not been described, and the regulation of expression may not be at the level of direct transcriptional control, a proposition supported by the smaller magnitude of induction by progestins compared with that reported to be transcriptionally mediated (28, 36). Although progestin action is acknowledged to be mediated almost entirely by the transactivating function of the hormone-bound progesterone receptor (4), steroidal stabilization of mRNA (9, 56) provides an alternate mechanism. Finally, steroidal regulation of other signal transduction pathways which in turn regulate oncogene expression is possible and is supported by the modulation of cyclic AMP activity by mitogenic steroids in some systems (60) and by increases, albeit delayed, in phosphoinositide turnover in estrogen-treated MCF-7 cells (21). A link between steroid receptors and other signal transduction pathways is also suggested by evidence for regulation of estrogen receptor mRNA expression by a phorbol ester which activates protein kinase C (42). Recent reports have demonstrated mutual cross-repression of transcription between the glucocorticoid receptor and the AP-1 transcription factor or its components, Jun and Fos (16, 34, 45, 64, 74); other steroid receptors may also participate in similar cross-talk between signal transduction pathways (64). While further investigation is required to establish the mechanisms for the progestin modulation of proto-oncogene expression and its role in the regulation of breast cancer cell proliferation, regulation over a time course of less than 1 h is among the most rapid changes of gene expression upon steroid treatment yet reported and suggests that c-*myc* may prove to be a valuable marker of progestin action in breast cancer cells.

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