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Post-Transcriptional Control of the Onset of DNA Synthesis by an Insulin-Like Growth Factor

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The control of eucaryotic cell proliferation is governed largely by a series of regulatory events which occur in the G1 phase of the cell cycle. When stimulated to proliferate, quiescent (G0) 3T3 fibroblasts require transcription, rapid translation, and three growth factors for the growth state transition. We examined exponentially growing 3T3 cells to relate the requirements for G1 transit to those necessary for the transition from the G0 to the S phase. Cycling cells in the G1 phase required transcription, rapid translation, and a single growth factor (insulin-like growth factor [IGF] I) to initiate DNA synthesis. IGF I acted post-transcriptionally at a late G1 step. All cells in the G1 phase entered the S phase on schedule if either insulin (hyperphysiological concentration) or IGF I (subnanomolar concentration) was provided as the sole growth factor. In medium lacking all growth factors, only cells within 2 to 3 h of the S phase were able to initiate DNA synthesis. Similarly, cells within 2 to 3 h of the S phase were less dependent on transcription and translation for entry into the S phase. Cells responded very differently to inhibited translation than to growth factor deprivation. Cells in the early and mid-G1 phases did not progress toward the S phase during transcriptional or translational inhibition, and during translational inhibition they actually regressed from the S phase. In the absence of growth factors, however, these cells continued progressing toward the S phase, but still required IGF at a terminal step before initiating DNA synthesis. We conclude that a suboptimal condition causes cells to either progress or regress in the cell cycle rather than freezing them at their initial position. By using synchronized cultures, we also show that in contrast to earlier events, this final, IGF-dependent step did not require new transcription. This result is in conrast to findings that other growth factors induce new transcription. We examined the requirements for G1 transit by using a chemically transformed 3T3 cell line (BPA31 cells) which has lost some but not all ability to regulate its growth. Early- and mid-G1-phase BPA31 cells required transcription and translation to initiate DNA synthesis, although they did not regress from the S phase during translational inhibition. However, these cells did not need IGF for entry into the S phase.

Eucaryotic cell growth is controlled by regulatory events which occur before the onset of DNA synthesis (2, 14, 23). The presynthesis or G1 phase of the cell cycle is generally viewed as a period during which cells prepare to initiate a round of DNA replication and cell division. In addition, cells in the G1 phase assess the feasibility of these processes in regard to the extracellular environment.

Much of our understanding of the nature of growth regulation derives from studies with cultured murine 3T3 fibroblasts. These established, nontumorigenic embryonic cells show a stringent degree of growth control in culture, depending on the environment into which they are placed (14, 16, 23, 34). They readily enter a reversible quiescent state when grown to confluency or deprived of serum factors. When deprived of serum, they readily resume proliferation after serum is restored. Moreover, 3T3 cells can be transformed to tumorigenicity by a variety of oncogenic chemicals and viruses. The resulting transformants show a spectrum of abnormalities or relaxations in their growth-regulatory properties.

The events regulating the emergence from quiescence and subsequent proliferation of 3T3 cells are organized sequentially, extending over ca. 12 h and concluding with the onset of DNA synthesis (S phase). Each identified event requires a growth factor (commonly supplied by serum) (12, 13, 39) or adequate supply of essential nutrients (12). In addition, both transcription and translation are necessary for stimulated, quiescent cells to enter the S phase (2, 3, 14, 38).

Biochemical markers that can be used to show progress toward the S phase are not readily quantitated. Thus, the position of cells with respect to the S phase is often indicated by the minimum time required to initiate DNA synthesis after the conditions are made optimal. The timing of growth factor requirements during the cell cycle can provide an understanding of how growth factor actions relate to the sequential biochemical events that regulate proliferation. Chronological position with respect to the onset of DNA synthesis is subject to various experimental perturbations, but remarkably consistent results are obtained by laboratories using the same cell lines and media.

The first proliferative event after cells in the G0 phase are stimulated is called competence and is dependent on platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or other competence factors (24, 34). Quiescent cells require 12 h to reach the S phase after serum is added, as do competent cells. However, competent cells no longer need PDGF. Cells can progress to the next control point, V, if they are provided with epidermal growth factor (EGF) and a very low concentration of somatomedin C (Sm-C) (21). Cells reach V ca. 6 h before reaching S. Progression past the V point depends on a rapid rate of protein synthesis (29), and thus on an adequate supply of amino acids (40), and on only one growth factor, Sm-C (21). After cells reach a third control point, R (22), ca. 2 h before S, they no longer require

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rapid protein synthesis or serum factors to initiate DNA synthesis (29, 44). A final control point, W, has also been reported (10, 25); some cells that are deprived of growth factors just before reaching the S phase cannot initiate DNA synthesis, but restoration of serum factors allows DNA synthesis to occur after only a short time.

Exponentially growing cells also need the competence event, and it is accomplished in the previous cycle (34, 35). It is not known when EGF and Sm-C exert their actions during exponential growth.

In this paper, we show that the only growth factor needed for entry into the S phase by cycling 3T3 cells in the G1 phase is insulin or insulin-like growth factors (IGFs), of which Sm-C (IGF I) is the most potent. IGF action could not be mimicked by cyclic AMP (cAMP) derivatives. When briefly deprived of IGF, cells progressed toward S for a few hours and initiated DNA synthesis very quickly after restoration of the growth factor. Longer IGF starvation caused the cells to enter the G0 phase. By contrast, brief inhibition of transcription or translation delayed cells in the G1 phase from entering the S phase by an interval equal to or greater than the interval of inhibition. IGF appeared to act late in the G1 phase; IGF action did not require new transcription. Finally, G1 transit by a chemically transformed 3T3 derivative was dependent on both transcription and translation, but was independent of IGF.

MATERIALS AND METHODS

Cell culture. Murine 3T3 fibroblasts, clone A31-CL7 (A31 cells), and benzo[*a*]pyrene-transformed 3T3 cells (BPA31 cells) were obtained (15), subcloned, and grown as described previously (3). New cultures were started from frozen stocks every 6 to 8 weeks. Each stock was determined to be free of mycoplasma contamination by using ratios of incorporated [^{3}H]uridine and [^{3}H]uracil (36).

Nuclear labeling and autoradiography. For exponential growth experiments, cells were plated at $3.5 \times 10^3/cm^2$ in 16-mm multiwell plates in Dulbecco modified Eagle medium (DME) plus glutamine (4 mM) and 10% calf serum (complete medium). The medium was aspirated 48 h later and replaced with 0.5 ml of medium containing 2 μCi of [*methyl*-^{3}H]thymidine per ml (ca. 80 Ci/mmol). For treatment with medium lacking serum, the cells were first washed twice with 2 ml of DME and then incubated with DME plus glutamine with or without growth factors, as appropriate. Inhibitors were added in complete medium and were removed by washing twice with the same medium.

For synchronous growth experiments, cells were plated at $7 \times 10^3/cm^2$ as described above. After 24 h they were washed with DME and incubated in 0.5% calf serum for 48 h. The cells were then quiescent; they were stimulated by addition of fresh complete medium.

At the indicated times, radiolabel incorporation was stopped by addition of 1 M ascorbic acid; at the end of the experiment, wells were processed for autoradiography and stained with Giemsa as previously described (3). The percent labeled nuclei was determined by counting 200 to 800 cells under 400× magnification.

Flow microfluorimetry. Cells were plated at $3 \times 10^3/cm^2$ in 60-mm culture dishes; 48 h later they were washed with DME and incubated in serum-free medium for 12 h, followed by incubation in 10% calf serum as described below. At the indicated times, they were washed with hypotonic propidium iodide and prepared and analyzed as previously described (29, 44).

RNA fractionation. Total RNA was isolated by a modification of the methods of Cox (8) and Strohman et al. (41). Polyadenylated [poly(A)$^+$] and nonpolyadenylated [poly(A)$^-$] fractions were separated by two passes through an oligodeoxythymidylate-cellulose column by the method of Bantle et al. (1).

Materials. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass.; non-cytotoxic insulin, EGF, FGF, and multiplication-stimulating activity (MSA) factors were purchased from Collaborative Research, Inc., Waltham, Mass.; cycloheximide (CHM), 5,6-dichlororibofuranosylbenzimidazole (DRB), prostaglandin F2α, dexamethasone, hydrocortisone, human thrombin, and transferrin were from Sigma Chemical Co., St. Louis, Mo. Sm-C (containing no detectable IGF II) was generously provided by R. Furlanetto, MSA (pooled fraction no. 3) was from J. Florini, and PDGF was from C. Stiles.

RESULTS

Insulin is the only growth factor needed by G1-phase cells for initiation of DNA synthesis. A31 cells are untransformed 3T3 fibroblasts. They have a 16 to 17-h doubling time and arrest growth (in the G0 phase) after 48 h of culture with 0.5% serum (3, 28). G1 transit by most exponentially growing A31 cells was a serum-dependent process (Fig. 1). The cells were shifted to medium (DME plus glutamine) containing [^{3}H]thymidine. At various times (1 to 24 h) thereafter, isotope incorporation was stopped and the cells were processed for autoradiography. About 50% of the cells were synthesizing DNA at the start of the experiment. In control cultures (containing 10% serum), the percent labeled nuclei increased to 98% as cells initially in the G1, M, and G2 phases entered the S phase. In this way we determined the kinetics of entry into the S phase by those exponentially growing cells which originally were distributed in the G1 phase, 38% as estimated from DNA histograms obtained by flow microfluorimetry (data not shown).

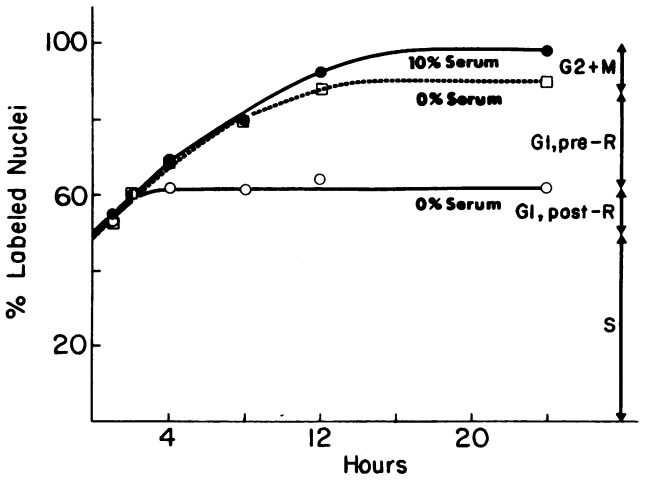

FIG. 1. Exponentially growing A31 cells require serum factors for G1 transit through R. Exponentially growing A31 or BPA31 cells were shifted to control medium (10% serum) or serum-free medium, both containing [^{3}H]thymidine. At the indicated times, radiolabel incorporation was stopped. Cells were processed for autoradiography, and the percent labeled nuclei was determined as described in the text. Washing did not affect the control curve. From these data, we estimate that in an exponentially growing population, 50% of the cells are in the S phase and 12% have passed the R point. Symbols: ●, A31 and BPA31 cells, control (10% serum); ○, A31 cells, serum-free medium; □, BPA31 cells, serum-free medium.

When shifted to serum-free medium, 10% of the A31 cells entered the S phase at the same rate as the control cells, during the first 2 h only. Therefore, serum deprivation did not impede progress into the S phase of cells which at the start of the experiment were beyond the serum-related R point, which occurred ca. 2 h before S. The R point was shown to be independent of the initial serum concentration and is therefore independent of factors adsorbed to the cells (45). The remaining cells were completely prevented from entering the S phase by removal of serum. We conclude that A31 cells that have not reached the R point require serum factors to initiate DNA synthesis.

The tumorigenic, benzo[*a*]pyrene-transformed BPA31 cells (15) behaved very differently. All the BPA31 cells in the G1 phase initiated DNA synthesis on schedule in the absence of all serum factors (Fig. 1). These transformed cells, like A31 cells, enter the G0 phase after culture in serum-deficient medium; however, they do so with difficulty, requiring more time (72 h) and a lower serum concentration (0.2%) (3, 28).

We sought to identify the factor(s) in serum required by A31 cells for G1 transit and passage through the R point. Exponentially growing cultures were shifted into serum-free medium containing individual factors which have been reported as stimulating the growth of 3T3 cells and other fibroblasts. These included insulin (1 μg/ml) (14, 19, 37), EGF (50 ng/ml) (4), FGF (50 ng/ml) (15, 33), PDGF (75 U/ml) (34), dexamethasone or hydrocortisone (30 ng/ml) (16, 33), prostaglandin F2α (400 ng/ml) (18, 19), thrombin (100 ng/ml) (9), and transferrin (5 μg/ml) (37). The progress into the S phase was monitored (Fig. 1).

Insulin was the only one of these factors which stimulated all of the cells in the G1 phase to initiate DNA synthesis (Fig. 2). In serum-free medium containing only insulin, 85 to 90% of the cells had synthesized DNA by 24 h after the start of the experiment. This fraction is at least equal to the percentage of cells initially in the S and G1 phases. Moreover, the insulin-treated cells and the control cells in 10% serum entered the S phase with very similar kinetics.

Insulin acts through the IGF I receptor. Insulin at physiological concentration is not mitogenic for fibroblasts. At high doses, it binds to IGF receptors, which do promote proliferation when occupied. Because the concentration of insulin used here (1 μg/ml) was hyperphysiological, it seemed likely that this hormone was acting through an IGF receptor (6). Serum contains ca. 1 ng of insulin per ml (26) and 200 ng of Sm-C per ml, which is mostly bound to transport proteins (6). We therefore determined the dose response of G1 transit to insulin and two IGFs, Sm-C and MSA, to identify the receptor through which insulin was controlling the onset of DNA synthesis (Fig. 3). Sm-C and MSA bind most tightly to the IGF I and IGF II receptors, respectively (6). Sm-C was clearly the most potent of these hormones, with maximum stimulatory effect at the lowest concentration tested (1 ng/ml); significant stimulation was observed with insulin and MSA at moderate concentrations (30 ng/ml). We conclude that the serum factor responsible for stimulating G1 transit by exponentially growing A31 cells is an IGF, probably Sm-C (IGF I). However, insulin was used for most experiments becuase of the cost and scarcity of Sm-C.

Neither insulin (1 μg/ml) nor MSA (100 ng/ml) alone stimulated either A31 or BPA31 cells to initiate DNA synthesis from quiescence (data not shown).

Insulin acts late in G1. Sm-C (or insulin at hyperphysiological concentrations) has been reported to be the only growth factor required during the final 6 h before the onset of DNA synthesis after stimulation from quiescence (21). Our results suggest that these last 6 h are equivalent to the G1 period of exponentially growing cells. It is not known whether Sm-C is required during the full 6-h period or whether its removal arrests cells statically or allows them to move backward or forward with respect to the time required for entry into the S phase after the factor is restored.

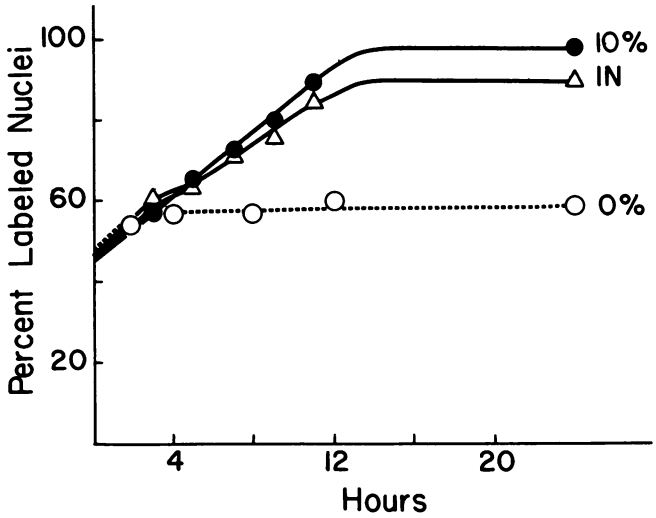

FIG. 2. Insulin is the only growth factor needed by A31 cells in the G1 phase for entry into the S phase. Exponentially growing cells were shifted into control medium (10% serum) or serum-free medium containing 1 μg of insulin (IN) per ml. Details are as described in the legend to Fig. 1. Symbols: ●, control medium; △, serum-free medium (1 μg of insulin per ml); ○, serum-free medium (no supplement) (data replotted from Fig. 1).

When quiescent, serum-stimulated 3T3 cells are treated with CHM, they arrest in phase if transformed and actually regress if untransformed (3). This regression is attributed to the decay of a protein with a short (2.5-h) half-life (3). We observed the same kinetics for exponentially growing A31 and BPA31 cells (Fig. 4). Administration of a set of CHM pulses (0.1 μg/ml, 60% inhibitory) did not prevent the cells initially located past R from entering the S phase. However, the CHM pulses delayed the A31 cells initially located before R; they were delayed by intervals longer than the length of the CHM pulses. We interpret the extra delays as indicative of the decay of a labile protein that is needed for passage through R. Presumably, other processes needed for G1 transit continue in the presence of CHM so that the cells treated with CHM for longer times enter the S phase at a faster rate than untreated cells or cells treated for shorter times. From these data we calculate a 2.7-h half-life for the labile factor, consistent with our previous experiments (3). CHM pulses also delayed exponentially growing BPA31 cells from entering the S phase. However, the delays were equal to the length of the CHM pulses (data not shown), also in agreement with our previous findings with synchronized cultures (3).

In similar experiments we used serum deprivation to determine whether removal of serum (specifically IGF) caused cells to arrest, regress from the G1-S boundary, or continue progress toward the S phase. Exponentially growing A31 cells were shifted into serum-free medium for a few hours, after which insulin was added to the culture. We know that only cells beyond R enter the S phase in serum-free medium (Fig. 1 and 2). If cells initially at stages before R were arrested there and could not pass this point in serum-

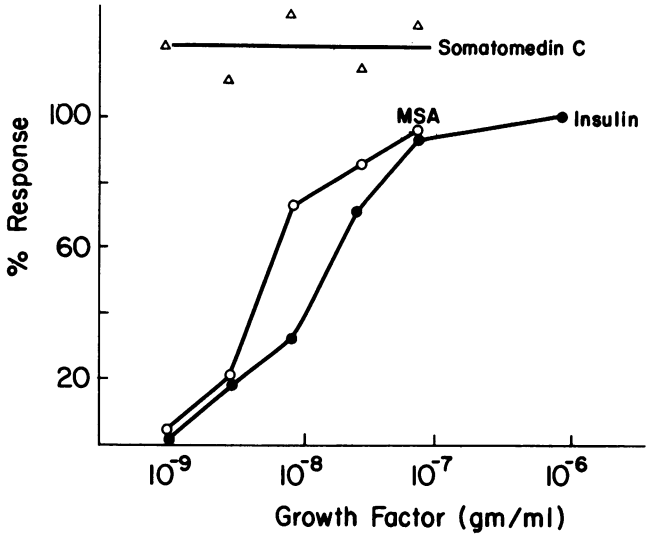

FIG. 3. G1 transit by exponentially growing cells; dose response to insulin, MSA, and Sm-C. Exponentially growing cells were shifted into serum-free medium containing bovine serum albumin (1 mg/ml) with or without the indicated concentrations of growth factor, all containing [^{3}H]thymidine. After 24 h the cells were fixed for autoradiography. The labeling index in the absence of any growth factor (ca. 57%) is plotted as 0% response. The labeling index in the presence of 1 μg of insulin per ml (ca. 78%) is plotted as 100% response. Bovine serum albumin at 1 mg/ml was slightly inhibitory, since these values were 5 to 7% lower than those observed in its absence.

free medium, then after subsequent insulin addition the rise in the labeling index should be delayed for at least 2 h (as in Fig. 4), the time required for the arrested cells to move from R into S. If cells in serum-free medium regressed toward the early G1 phase (away from the G1-S boundary), the labeling index increase should be delayed with kinetics similar to those shown in Fig. 4. If, on the other hand, cells in serum-free medium progressed toward the S phase and were blocked at W, the labeling index should increase almost immediately after insulin is added.

After insulin was added, cells briefly starved of serum initiated DNA synthesis almost immediately, with initial kinetics very similar to those in the control culture (Fig. 5). Thus, cells initially located before R at the start of the starvation period were not arrested at the R point in serum-free medium. Rather, these cells advanced toward the G1-S boundary and were able to complete all except the final step(s) necessary for the initiation of DNA synthesis; this final step(s) specifically required insulin or, more accurately, IGF I. Therefore, pre-R cells in the G1 phase arrested close to the G1-S boundary (at W) in serum-free medium until they were given insulin.

The cycling pre-R-point cells exposed to serum-free medium gradually became quiescent. The final (24-h) labeling index decreased as the duration of the serum-free interval increased (Fig. 5, inset). Half of the initially responsive cells became unresponsive to insulin after 5 to 6 h. These unresponsive cells were in a viable, quiescent state. After 12 h in serum-free medium, more than 80% of the population had a

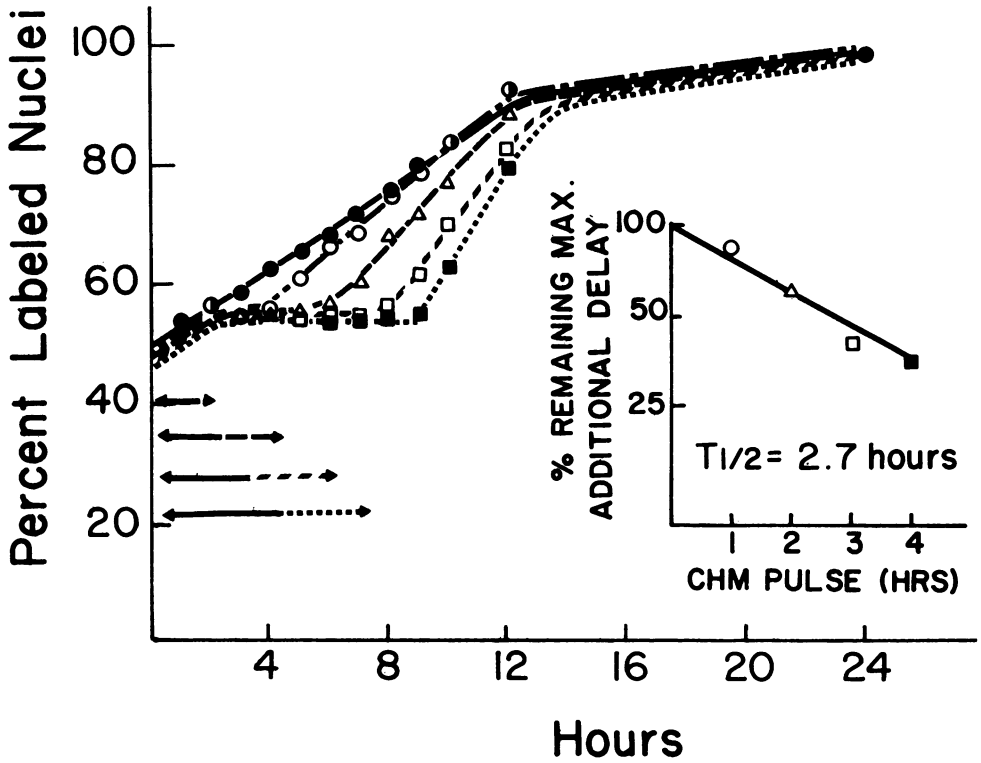

FIG. 4. A31 cells regress to early G1 in the presence of CHM. Exponentially growing cells were shifted to control medium (10% serum, solid line) or to medium containing 10% serum and 0.1 μg of CHM per ml (broken lines). At the indicated intervals (1 to 4 h), the CHM-containing medium was removed, the cells were washed twice with 10% serum, and 10% serum without drug was restored to the culture. The initiation of DNA synthesis over time was monitored by autoradiography as described in the legend to Fig. 1. The lengths of the CHM pulses are indicated by solid arrow-headed lines, and the lengths of the additional delays in the onset of DNA synthesis are indicated by the broken arrow-headed lines. (Inset) Calculated half-life for the decay process causing the G1 regression. The log of the percent maximum delay remaining after the CHM pulse (the maximum additional delay minus the additional delay at a given CHM pulse) is plotted against the length of the CHM pulse as previously described (3).

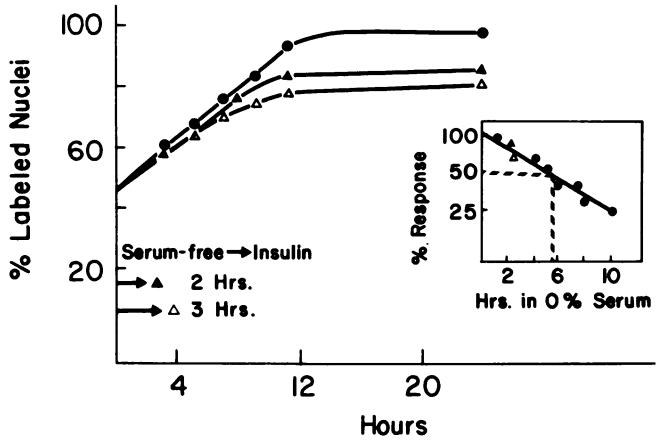

FIG. 5. Cells progress to the G1-S boundary in the absence of serum and initiate DNA synthesis when insulin is provided. Exponentially growing cells were shifted to control medium (10% serum, ●) or serum-free medium (△, ▲) for 2 (▲) or 3 (△) h. At the end of the serum-free incubation, the medium was changed to serum-free medium plus 1 μg of insulin per ml. The ability of cells to initiate DNA synthesis over time was monitored by autoradiography as described in the legend to Fig. 1. (Inset) In a similar, separate experiment, cells were incubated in serum-free medium for intervals ranging from 1 to 10 h. After incubation, serum-free medium plus 1 μg of insulin per ml was added until 24 h after the start of the experiment. The labeling index was determined at this time. The 24-h labeling index of cells kept in serum-free medium throughout the experiment is defined as 0% response; 100% response was the 24-h labeling index of cells kept in serum-free medium plus 1 μg of insulin per ml throughout the experiment. Shown is a log plot of this response versus the duration of the serum-free intervals. In serum-free medium, the ability to respond to insulin decayed with a half-life of ca. 5.5 h (broken line).

G1 DNA content, as estimated by flow microfluorimetry (data not shown); 10.2% were in the S phase as measured by a 1-h pulse with [^{3}H]thymidine. These serum-starved cells, when given 10% serum, began to synthesize DNA about 12 h afterwards, which corresponds to the length of the prereplicative phase during emergence from quiescence (25). At 12 h after the serum was added, 12.6% of the cells were in the S phase. Twenty hours after serum addition, 86.9% of the cells were in the S phase, and most of the cells had a higher DNA content than they did in the G1 phase as measured by flow microfluorimetry (data not shown).

Post-transcriptional action of Sm-C. The undelayed stimulation by insulin of entry into the S phase (Fig. 5) suggested that IGF I might act on cells near the G1-S boundary by a rapid, and thus possibly post-transcriptional or post-translational, mechanism. To test this idea, we examined the effects of transcription and translation inhibitors on the ability of insulin to promote the initiation of DNA synthesis by G1-phase cells.

DRB is an inhibitor of transcription with some specificity towards hnRNA synthesis (42). DRB was added to cultures at a concentration (15 μg/ml) that blocks the transcription induced by PDGF in 3T3 cells (38); it was sufficient to inhibit [^{3}H]uridine incorporation into total RNA and into poly(A)$^+$ RNA by about 50 and 80%, respectively (Table 1).

G1 transit by cycling A31 and BPA31 cells was substantially retarded by DRB (Fig. 6). In exponentially growing cultures shifted to 10% serum (Fig. 6) or serum-free medium plus insulin (data not shown), DRB only gradually affected the entry of cells into the S phase after 3 to 4 h; few of the remaining cells entered the S phase even after 24 h. Therefore, DRB did not inhibit insulin action on cells within a few hours of the G1-S boundary; it did inhibit transit by early G1-phase cells. When pulsed with DRB for 2 h, exponentially growing A31 cells in the early G1 phase were delayed in initiating DNA synthesis by 2 h (data not shown). Therefore, exponentially growing late-G1-phase cells were relatively insensitive to transcriptional inhibition; transcription was more important in the early G1 phase.

TABLE 1. Inhibition of RNA synthesis by DRB[a]

RNA fraction	[^{3}H]uridine incorporation		
	Amt (10^6 cpm)		% of control
	Without DRB	With DRB	
Total	8.8	3.8	43
Poly(A)$^-$	6.2	3.0	45
Poly(A)$^+$	0.81	0.19	23

[a] Exponentially growing cells were plated at 2.8×10^3/cm^2; 60 h later they were incubated with 0.075% dimethyl sulfoxide (DMSO) (controls) or 15 μg of DRB per ml in DMSO (0.075%) for 10 min. [^{3}H]uridine (3.75 μCi/ml, 26.5 Ci/mmol) was then added for 3 h. Total RNA was isolated and fractionated into poly(A)$^-$ and poly(A)$^+$ species as described in the text.

We next used synchronized cells to apply the inhibitor to a cohort of cells close to the IGF I-sensitive point. Quiescent A31 cells were stimulated to progress toward the S phase by serum readdition. After 12 h, as the first cells entered the S phase, the culture was shifted to control (10% serum) or serum-free medium with or without insulin (Fig. 7). Control cells showed 98% labeled nuclei 24 h after the shift, whereas cells in serum-free medium showed about 30% labeled nuclei. Insulin added to the serum-free medium stimulated ca. 75% of the cells to initiate DNA synthesis.

When the shift medium contained insulin, DRB did not

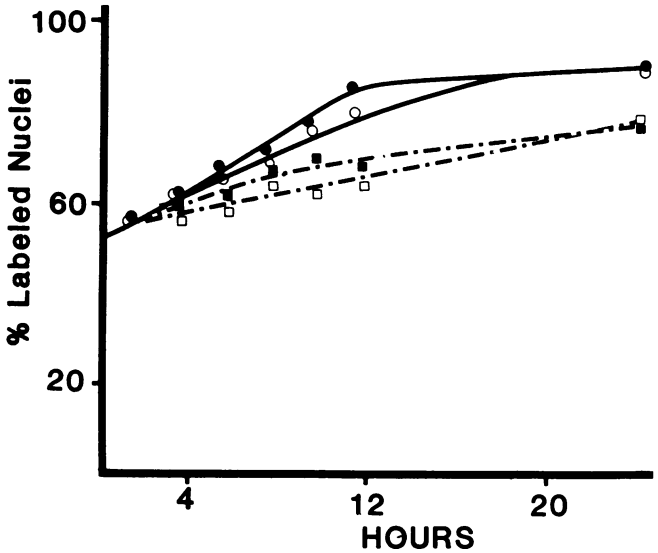

FIG. 6. G1 transit is retarded by DRB. Exponentially growing A31 and BPA31 cells were shifted to control medium (10% serum) or DRB (15 μg/ml, added as a solution in DMSO) containing [^{3}H]thymidine. At the indicated times, cells were processed for autoradiography as described in the legend to Fig. 1. Control media contained an equivalent amount of DMSO (0.075%). This concentration of DMSO slightly retarded G1 transit by both exponential and synchronous cultures. Symbols: ○, A31 cells, control medium; ●, BPA31 cells, control medium; □, A31 cells, DRB medium; ■, BPA31 cells, DRB medium.

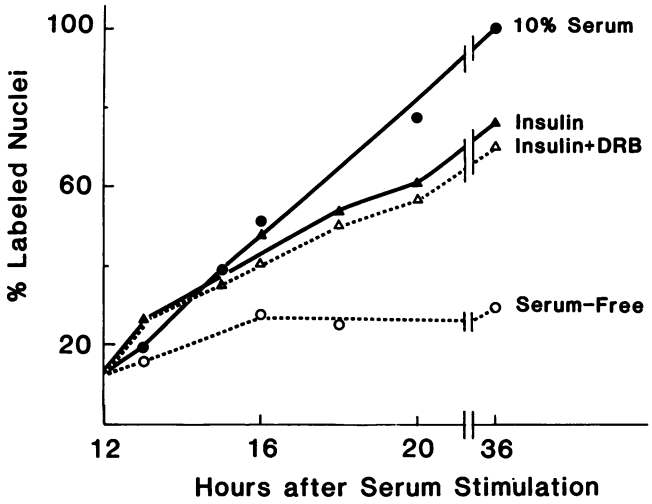

FIG. 7. Insulin stimulation of the initiation of DNA synthesis is not inhibited by DRB. Quiescent cells were stimulated as described in the text. At 12 h after stimulation, the cells were washed with DME and incubated in medium containing [^{3}H]thymidine. At the indicated times, radiolabeled incorporation was stopped, and the cells were processed for autoradiography. Symbols: ●, 10% serum; ▲, serum-free medium plus 1 μg of insulin per ml; △, serum-free medium plus insulin and 15 μg of DRB per ml; ○, serum-free medium only.

inhibit the cells from initiating DNA synthesis; the kinetics were similar for cells treated with insulin alone. The final labeling index was 69%.

A second type of experiment was done in which quiescent cells were stimulated for 12 h and then accumulated near the G1-S boundary by being shifted into serum-free medium for 6 h (W-point arrest [25]). They were then shifted into experimental medium (Fig. 8). As shown in Fig. 7, 28% of the cells receiving neither serum nor insulin entered the S phase within 24 h, whereas 95% of the cells receiving 10% serum did so. About 68% of the cells initiated DNA synthesis when DRB was included with the 10% serum. Insulin in serum-free medium permitted 65% of the cells to initiate DNA synthesis in the absence of the inhibitor and 61% to do so in its presence. Thus, DRB did not prevent entry into the S phase by cells that required only insulin. These results show that transcription is unnecessary for initiation of DNA synthesis by G1-phase cells close to the S phase.

By contrast, translation is necessary for the initiation of DNA synthesis by cells close to the S phase. A31 cells were prepared as described for Fig. 6 except that CHM (0.5 μg/ml, 90% inhibitory) was added in place of DRB. CHM inhibited insulin-dependent initiation of DNA synthesis (35% labeled nuclei at 24 h) in cells stimulated for 12 h as in Fig. 6. We could not determine from this experiment whether the insulin-requiring step, or some other entry step, is sensitive to CHM.

Post-transcriptional control of growth can involve protein phosphorylation, possibly by a cAMP-dependent kinase (30). Agents which raise intracellular cAMP levels were unable to substitute for insulin in stimulating the initiation of DNA synthesis in either exponential or synchronized cultures. When dibutyryl-cAMP (1 mM), 8-bromo-cAMP (0.5 mM), or theophylline (0.25 mM) was added, the labeling index was similar to that obtained in serum-free medium alone (data not shown). This result contrasts with the ability of choleragen, which raises cAMP levels, to replace EGF (44).

DISCUSSION

We conclude that G1 transit by exponentially growing cells requires only IGF I and that this growth factor probably acts on cells close to the G1-S boundary by a post-transcriptional mechanism. Post-transcriptional activity is a novel feature of growth factors in general. IGF I action can constitute a terminal controlling event for the onset of DNA synthesis. Cells which do not soon complete this terminal step enter quiescence.

To relate our findings to information on control points (Fig. 9), we considered cells in exponential growth, initially before the S phase. The cells located beyond the R point did not need any growth factors (or rapid protein synthesis) to enter the S phase on schedule. All cells in the G1 phase (equivalent to being beyond the V point [21]) also entered the S phase on schedule if provided with IGF I (or insulin) as the sole growth factor. In the absence of IGF I for a few hours, cells between the V and R points progressed toward the S phase, and some reached the W point near the G1-S boundary but did not initiate DNA synthesis. That is, cells at W appear to be ready to start DNA synthesis except that they require IGF I.

Unlike PDGF (7, 38), IGF action did not require rapid new mRNA synthesis to stimulate DNA synthesis, as shown by the failure of the relatively specific RNA synthesis inhibitor DRB to block entry into the S phase (42). When cells that had accumulated at the G1-S boundary after a 6-h serum starvation were stimulated with insulin, DRB did not inhibit the initiation of DNA synthesis. About 30% of the cells in these synchronized cultures were not stimulated by insulin, and we presume these to be early-G1-phase (or even pre-V-point) cells. This same fraction could not enter the S phase when given 10% serum plus DRB. DRB also delayed G1 transit by chicken embryo fibroblasts (5). Inhibitor studies of quiescent, serum-starved murine AKR-2B cells have similarly shown that hnRNA synthesis is required for entry into the S phase in the first few hours after serum stimulation, but not in the last few hours (43).

DRB is a relatively strong inhibitor of mRNA synthesis. We used the same DRB concentration which prevented the induction of competence by PDGF in A31 cells (38); this concentration did not inhibit the effect of insulin. In addition, [^{3}H]uridine incorporation into poly(A)-selected RNA was reduced by about 80% relative to uninhibited cultures. DRB can also inhibit uridine uptake, but this cannot account for

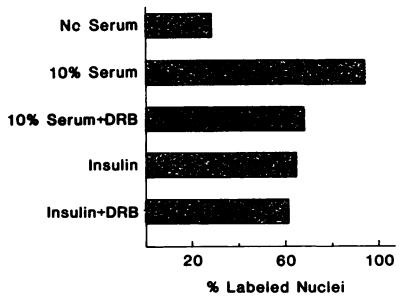

FIG. 8. Effect of DRB on initiation of DNA synthesis by cells at the G1-S boundary. Cells were arrested close to the G1-S boundary. They were made quiescent and stimulated as described in the text. At 12 h after stimulation, the cells were washed and incubated in serum-free medium plus [^{3}H]thymidine for 6 h. The serum-free medium was then replaced by serum-free medium plus 15 μg of DRB per ml. After 10 min, the medium was again replaced with the indicated experimental media containing [^{3}H]thymidine. Labeled nuclei were counted 24 h later.

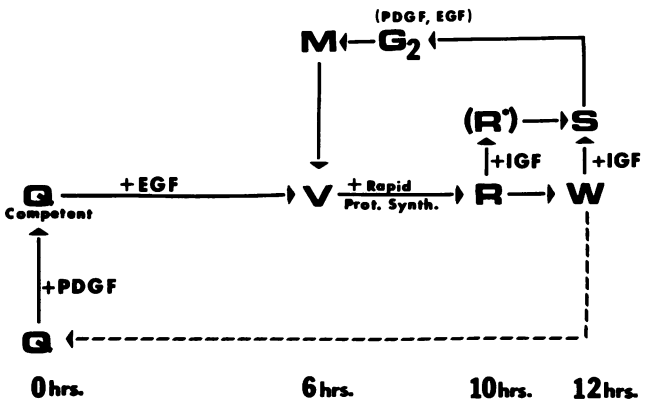

FIG. 9. Growth control points in the 3T3 cell cycle. The initiation of DNA synthesis requires a minimum of 12 h after stimulation of quiescent cells and 6 h after mitosis in cycling cells. Quiescent cells are made competent by PDGF or other competence factors, after which they progress to the V point in the presence of IGF and EGF. Cycling cells also arrive at V after mitosis; PDGF (and probably EGF) acts in the previous cycle. Cells at V require a rapid rate of protein synthesis to reach the R point. Cells which have reached R in the presence of IGF achieve an activated-state R (R*), initiate DNA synthesis 2 h later, and require neither growth factors nor a rapid rate of protein synthesis to do so. In the absence of IGF, cells pass the R point and reach the G1-S boundary (W). If provided with IGF, they enter the S phase nearly immediately; if not, they enter quiescence (Q).

the differential inhibition of mRNA versus rRNA synthesis. Furthermore, at this DRB concentration, total uridine uptake is inhibited to approximately the same extent as total RNA synthesis (42). Inhibition of mRNA synthesis may be underestimated, since even after two passes through an oligodeoxythymidylate column, some contamination of poly(A)$^+$ RNA with rRNA is likely. Nonetheless, 80% apparent inhibition of poly(A)$^+$ RNA synthesis did not appreciably delay or inhibit the initiation of DNA synthesis stimulated by IGF. This level of inhibition does retard progress through the S phase (as it does progress through the G1 phase) since mRNA synthesis is needed throughout the S phase. Nuclear labeling scores only entry into the S phase. At 80% inhibition, there is apparently sufficient histone mRNA to permit some DNA synthesis.

Cells at the W point were only moderately stable, since their ability to enter the S phase after addition of IGF deteriorated with a half-life calculated at 5 to 6 h. This is similar to the half-life reported by Das (10) for cells stopped at the G1-S boundary by hydroxyurea. These cells gradually lost their ability to enter S after removal first of the mitogenic stimulus (15 or 2% serum plus EGF) and later of hydroxyurea. Our results indicate that cells in serum-free medium leave the cycle from W to enter the G0 (quiescence) phase.

IGF appears to act on G1 cells at different times depending on the growth conditions. A cell which has passed R (2 h before the S phase) in the presence of serum, having accumulated a critical level of labile protein (29), no longer requires IGF. The serum independence which cells develop at the R point could be due to a biochemical change delivered by IGF to cells that reach R. Cells that have reached R in the absence of serum would not have undergone this change and so would still require IGF to enter the S phase. Alternatively, it could take 2 h to exhaust the IGF effect after serum is removed. This seems unlikely since the location in time of the R point determined by CHM (rapid) treatment and by serum removal is very similar (see also references 3, 20, and 43), and the R point location is independent of the initial serum concentration (45). The nature of this biochemical change is unknown; it may act directly on the replication complex (27). Protein phosphorylation is a good candidate (31), and IGF I binding stimulates receptor phosphorylation (17, 32). Our results indicate that such phosphorylation must be cAMP independent.

Little is known about the relationship between transformation and the IGF requirement for cell growth. The post-transcriptional action of IGF suggests a novel mechanism for growth factor action and transformation. Genetically diminished IGF dependence for the initiation of DNA synthesis should diminish growth control by serum factors. Examples of a relationship between an IGF requirement and transformation exist. A human fibrosarcoma line produces a peptide with insulin-like activity (11). The BPA31 cells traversed the G1 phase and initiated DNA synthesis during exponential growth in the absence of all serum factors, unlike the untransformed 3T3 cells. Medium conditioned by BPA31 cells did not stimulate G1 transit by A31 cells (unpublished data). Thus, this transformation eliminated the IGF requirement for G1 transit. Our finding presents an opportunity to study this post-transcriptional event in normal and aberrant growth control.

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LITERATURE CITED

1. **Bantle, J. A., I. H. Maxwell, and W. C. Hahn.** 1976. Specificity of oligo(dT)-cellulose chromatography in the isolation of polyadenylated RNA. Anal. Biochem. **72**:413–427.
2. **Baserga, R.** 1976. Multiplication and division of mammalian cells. Marcel Dekker, Inc., New York.
3. **Campisi, J., E. E. Medrano, G. Morreo, and A. B. Pardee.** 1982. Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. Proc. Natl. Acad. Sci. U.S.A. **79**:436–440.
4. **Carpenter, G., and S. Cohen.** 1979. Epidermal growth factor. Annu. Rev. Biochem. **48**:193–216.
5. **Chadwick, D. E., G. G. Ignotz, R. A. Ignotz, and I. Lieberman.** 1980. Inhibitors of RNA synthesis and passage of chick embryo fibroblasts through the G1 period. J. Cell. Physiol. **104**:61–72.
6. **Clemmons, D. R., and J. J. Van Wyk.** 1981. Somatomedin: physiological control and effects on cell proliferation. Handb. Exp. Pharmacol. **57**:161–208.
7. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of the gene sequences regulated by platlet-derived growth factor. Cell **33**:939–947.
8. **Cox, R. A.** 1968. The use of guanidinium chloride in the isolation of nucleic acids. Methods Enzymol. **12B**:120–129.
9. **Cunningham, D. D., K. D. Glenn, J. B. Baker, R. L. Summer, and D. A. Low.** 1979. Mechanisms of thrombin-stimulated cell division. J. Supramol. Struct. **11**:259–267.
10. **Das, M.** 1981. Initiation of nuclear DNA replication: evidence for formation of committed prereplicative cellular state. Proc. Natl. Acad. Sci. U.S.A. **78**:5677–5681.
11. **De Larco, J. E., and G. J. Todaro.** 1978. A human fibrosarcoma cell line producing multiplication stimulating activity (MSA) peptides. Nature (London) **272**:356–358.
12. **Eagle, H.** 1955. Nutritional requirements of animal cells. Science **122**:501–504.
13. **Hayashi, I., and G. Sato.** 1976. Replacement of serum by hormones permits growth of cells in a defined medium. Nature (London) **259**:132–134.
14. **Hochhauser, S. J., J. L. Stein, and G. S. Stein.** 1981. Gene

expression and cell cycle regulation. Int. Rev. Cytol. **71**:95–243.
15. **Holley, R. W., J. H. Baldwin, J. A. Kiernan, and T. O. Messmer.** 1976. Control of growth of benzo(a)pyrene-transformed 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. **73**:3229–3232.
16. **Holley, R. W., and J. A. Kiernan.** 1974. Control of initiation of DNA synthesis in 3T3 cells: serum factors. Proc. Natl. Acad. Sci. U.S.A. **71**:2908–2911.
17. **Jacobs, S., K. C. Kull, H. S. Earp, M. E. Svoboda, J. J. Van Wyk, and P. Cuatrecasas.** 1983. Somatomedin-C stimulates the phosphorylation of the β-subunit of its own receptor. J. Biol. Chem. **258**:9581–9584.
18. **Jimenez de Asua, L., K. M. V. Richmond, and A. M. Otto.** 1981. Two growth factors and two hormones regulate initiation of DNA synthesis in cultured mouse cells through different pathways of events. Proc. Natl. Acad. Sci. U.S.A. **78**:1004–1008.
19. **Jimenez de Asua, L., K. M. V. Richmond, A. M. Otto, A. M. Kubler, M. K. O'Farrell, and P. S. Rudland.** 1979. Growth factors and hormones interact in a series of temporal steps to regulate the rate of initiation of DNA synthesis in mouse fibroblasts. Cold Spring Harbor Conf. Cell Proliferation **6**:403–424.
20. **Leof, E. B., J. J. Van Wyk, E. J. O'Keefe, and W. J. Pledger.** 1983. Epidermal growth factor (EGF) is required only during the traverse of early G1 in PDGF stimulated density-arrested Balb/c-3T3 cells. Exp. Cell Res. **147**:202–208.
21. **Leof, E. B., W. Wharton, J. J. Van Wyk, and W. J. Pledger.** 1982. Epidermal growth factor (EGF) and somatomedin C regulate G1 progression of competent Balb/c 3T3 cells. Exp. Cell Res. **141**:107–115.
22. **Pardee, A. B.** 1974. A restriction point for control of normal animal cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **71**:1286–1290.
23. **Pardee, A. B., R. D. Dubrow, J. L. Hamlin, and R. E. Kletzien.** 1978. Animal cell cycle. Annu. Rev. Biochem. **47**:715–750.
24. **Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1977. Induction of DNA synthesis in Balb/c 3T3 cells by serum components: reevaluation of the commitment process. Proc. Natl. Acad. Sci. U.S.A. **74**:4481–4485.
25. **Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1978. An ordered sequence of events is required before Balb/c-3T3 cells become committed to DNA synthesis. Proc. Natl. Acad. Sci. U.S.A. **75**:2839–2843.
26. **Price, P. J., and E. A. Gregory.** 1982. Relationship between *in vitro* growth promotion and biophysical and biochemical properties of the serum supplement. In Vitro (Rockville) **18**:576–584.
27. **Reddy, G. P. V., and A. B. Pardee.** 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc. Natl. Acad. Sci. U.S.A. **77**:3312–3316.
28. **Riddle, V. G. H., and D. M. Lehtomaki.** 1981. Growth arrest states of RNA virus and chemically transformed mouse cells. Cancer Res. **41**:1778–1783.
29. **Rossow, P. W., V. G. H. Riddle, and A. B. Pardee.** 1979. Synthesis of labile, serum-dependent protein in early G1 controls animal cell growth. Proc. Natl. Acad. Sci. U.S.A. **76**:4446–4450.
30. **Rozengurt, E., A. Legg, G. Strang, and N. Courtenay-Luck.** 1981. Cyclic AMP: a mitogenic signal for Swiss 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. **78**:4392–4396.
31. **Rubin, C. S., and O. M. Rosen.** 1975. Protein phosphorylation. Annu. Rev. Biochem. **44**:831–885.
32. **Rubin, J. B., M. A. Shia, and P. F. Pilch.** 1983. Stimulation of tyrosine-specific phosphorylation *in vitro* by insulin-like growth factor I. Nature (London) **305**:438–440.
33. **Rudland, P. S., W. Seifert, and D. Gospodarowicz.** 1974. Growth control in cultured mouse fibroblasts: induction of the pleiotypic and mutagenic responses by a purified growth factor. Proc. Natl. Acad. Sci. U.S.A. **71**:2600–2604.
34. **Scher, C. D., R. C. Shepard, H. N. Antoniades, and C. D. Stiles.** 1979. Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. Biochim. Biophys. Acta **560**:217–241.
35. **Scher, C. D., M. E. Stone, and C. D. Stiles.** 1979. Platelet-derived growth factor prevents G0 growth arrest. Nature (London) **281**:390–392.
36. **Schneider, E. L., E. J. Stanbridge, and C. J. Epstein.** 1974. Incorporatioon of ^{3}H-uridine and ^{3}H-uracil into RNA. A simple technique for the detection of mycoplasma contamination of cultured cells. Exp. Cell Res. **84**:311–318.
37. **Serrero, G. R., D. B. McClure, and G. H. Sato.** 1979. Growth of mouse 3T3 fibroblasts in serum-free, hormone supplemented media. Cold Spring Harbor Conf. Cell Proliferation. **6**:523–530.
38. **Smith, J. C., and C. D. Stiles.** 1981. Cytoplasmic transfer of the mitogenic response to platelet-derived growth factor. Proc. Natl. Acad. Sci. U.S.A. **78**:4363–4367.
39. **Stiles, C. D., G. T. Capone, C. D. Scher, H. N. Antoniades, J. J. Van Wyk, and W. J. Pledger.** 1979. Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc. Natl. Acad. Sci. U.S.A. **76**:1279–1283.
40. **Stiles, C. D., R. R. Isberg, W. J. Pledger, H. N. Antoniades, and C. D. Scher.** 1979. Control of the Balb/c-3T3 cell cycle by nutrients and serum factors: analysis using platelet-derived growth factor and platelet-poor plasma. J. Cell. Physiol. **99**:395–405.
41. **Strohman, R. C., P. S. Moss, J. Micou-Eastwood, and D. Spector.** 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. Cell **10**:265–273.
42. **Tamm, I., R. Hand, and L. A. Caliguiri.** 1976. Action of dichlorobenzimidazole riboside on RNA synthesis in L-929 and HeLa cells. J. Cell Biol. **69**:229–240.
43. **Wells, D. J., L. S. Stoddard, M. J. Getz, and H. L. Moses.** 1979. αAminitin and 5-fluorouridine inhibition of serum-stimulated DNA synthesis in quiescent AKR-2B mouse embryo cells. J. Cell. Physiol. **100**:199–214.
44. **Wharton, W., E. Leof, W. J. Pledger, and E. J. O'Keefe.** 1982. Modulation of the epidermal growth factor receptor by platelet-derived growth factor and choleragen: effects on mitogenesis. Proc. Natl. Acad. Sci. U.S.A. **79**:5567–5571.
45. **Yen, A., and A. B. Pardee.** 1978. Exponential 3T3 cells escape in mid-G1 from their high serum requirement. Exp. Cell Res. **116**:103–113.