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An ordered sequence of events is required before BALB/c-3T3 cells become committed to DNA synthesis

(transition probability/growth arrest points/platelets/plasma)

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ABSTRACT **An ordered sequence of events must be completed before cells become committed to synthesize DNA. A platelet-derived growth factor (PDGF), present in heated (100°) extracts of human platelets, induces density-inhibited BALB/c-3T3 cells to become competent to proliferate. Platelet-poor plasma induces these competent cells to leave the competence point, progress through G_0/G_1, and enter the S phase. Treatment of G_0-arrested, incompetent cells with plasma, before the addition of PDGF, did not shorten the latent period for DNA synthesis or increase the rate of entry into the S phase. Growth arrest points in the plasma-dependent progression sequence were detected in G_0/G_1. PDGF-treated competent cells were exposed to an optimal concentration of plasma (5%) for various lengths of time and were then transferred to medium lacking plasma; the subsequent readdition of plasma stimulated the cells to enter the S phase. The lag period until DNA synthesis, in such experiments, was dictated by the length of the initial exposure to plasma. PDGF-treated competent cells that were incubated with plasma for 5 hr during the initial exposure did not leave the competence point; they began DNA synthesis 12 hr after the readdition of plasma. However, a population of cells treated with plasma for 10 hr became arrested at a point 6 hr before DNA synthesis, whereas a population treated with plasma for 12–15 hr became arrested at a point immediately before DNA synthesis. Cells remained arrested at this latter point for as long as 24 hr, and these arrested cells were not committed to DNA synthesis. The addition of plasma induced immediate entry into the S phase with an apparent first-order rate of entry being determined by the plasma concentration. This plasma-dependent commitment (transition) to DNA synthesis was blocked by cycloheximide but not by hydroxyurea. Removal of the hydroxyurea allowed cells to enter the S phase synchronously in the absence of plasma.**

Serum induces quiescent, density-inhibited 3T3 cells to leave G_0, synthesize DNA, and replicate (1–3). There are two sets of growth factors in serum that control different phases of the cell cycle (4). One set is a heat-stable (100°) platelet-derived growth factor (PDGF) that is released into serum during the clotting process (5–9). PDGF induces BALB/c-3T3 cells to become competent to synthesize DNA. A second set of components, found in defibrinogenated platelet-poor plasma, allows competent cells to progress through G_0/G_1 and synthesize DNA (4). There is a lag of 12 hr from the time of plasma addition to the competent cells until the beginning of DNA synthesis (4). The events that occur during this lag period have not been defined.

Smith and Martin (10, 11) found that cells enter the S phase with first-order kinetics, and they proposed that the commitment of cells to synthesize DNA is a single, random event characterized by a first-order rate constant, termed the "transition probability." Because cells enter DNA synthesis with first-order kinetics, it was suggested that this rate-limiting commitment event divides the cell cycle into two fundamentally different parts: (*i*) a deterministic "B" state of relatively constant duration, including a portion of late G_1 phase and all of the S, G_2, and M phases; and (*ii*) a nondeterministic "A" state which includes early G_1 and, in growth-arrested cells, G_0 (10, 11). According to the transition probability model, some time after mitosis, a cell enters the A state. While in the A state, the cell's activity is not directed toward replication, and the cell does not progress toward division (10, 11). In support of the transition probability model, Brooks found that quiescent 3T3 (12) or BHK (13) cells entered the S phase with apparent first-order kinetics at 12–14 hr after stimulation with serum. Because the serum concentration determined the rate at which cells entered the S phase, it was suggested that serum commits cells to synthesize DNA by increasing the transition probability (12, 13). The 12- to 14-hr lag period until DNA synthesis was interpreted to be the minimal interval needed to increase the transition probability of a population of cells (12).

In accord with the transition probability model, BALB/c-3T3 cells continuously treated with both PDGF and platelet-poor plasma entered the S phase with apparent first-order kinetics at a rate that was determined by the plasma concentration (4). In order to evaluate more critically the transition probability model and define the events that occur prior to commitment, we have treated quiescent BALB/c-3T3 cells sequentially with platelet extract and platelet-poor plasma. Removal of these components at timed intervals caused cells to become arrested at distinct growth arrest points. Thus, there is an ordered sequence of PDGF- and plasma-dependent steps during the lag period before BALB/c-3T3 cells become committed to synthesize DNA. The serum-dependent events that occur prior to commitment have an ordered sequence that is directed toward cell replication. We also show that the actual commitment to DNA synthesis occurs after cells progress past a late growth arrest point in G_1. Cells that leave this arrest point begin to enter the S phase immediately at a rate determined by the plasma concentration.

MATERIALS AND METHODS

Cell Culture. All culture passages of density-inhibited BALB/c-3T3 (clone A31) cells require exposure to PDGF in order to become competent to synthesize DNA. Various passages appear to have different plasma requirements for progression through G_1 phase, however; a passage with a relatively high plasma requirement was used for this study. The stimu-

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Abbreviations: PDGF, platelet-derived growth factor; MSA, multiplication-stimulating activity.

lation of BALB/c-3T3 cells by platelet extract and platelet-poor plasma has been described (4). Briefly, cells were grown to confluence in 0.3-cm^2 microtest wells in Dulbecco's modified Eagle's medium containing 10% calf serum. The spent medium was removed and fresh medium (0.2 ml) containing platelet extract (4) was added for 5 hr as indicated to induce the cells to become competent to synthesize DNA. The platelet extract was removed and the cells were rapidly washed with 28 mM 2-mercaptoethanol in modified Eagle's medium and rinsed with medium alone. Medium containing platelet-poor plasma and [^{3}H]dThd (5 μCi/ml; 6.7 Ci/mmol) was added and the cells were incubated at 37°. At intervals, the plasma was removed, and the cells were washed once with medium and returned to medium containing [^{3}H]dThd. At intervals, this medium was removed and fresh medium containing platelet-poor plasma and [^{3}H]dThd was added. At appropriate times, the cells were fixed, washed with trichloroacetic acid, and processed for autoradiography as described (9). Between 200 and 400 cells were scored for each point.

Preparation of Platelet Extract and Platelet-Poor Plasma. The preparation of heat-treated (100°) extracts of human platelets has been described (8). The preparation of defibrinogenated platelet-poor plasma has been described (4). Only preparations that maintained cell viability without stimulating the growth of BALB/c-3T3 cells were used. Because these human blood preparations represent a potential source of hepatitis virus, no mouth pipetting was done and all samples were autoclaved before being discarded.

Inhibition Studies. BALB/c-3T3 cells were incubated in medium containing 5% plasma, [^{3}H]dThd, and 7.5 μg of cycloheximide (Sigma) per ml or 1.0 mM hydroxyurea (Sigma) for 12 hr. The concentration of cycloheximide chosen inhibits greater than 95% of protein synthesis in BALB/c-3T3 cells; the 1.0 mM hydroxyurea inhibits replicative DNA synthesis.

RESULTS

Competence Must Occur before Progression. Density-inhibited G_0-arrested BALB/c-3T3 cells were treated with an optimal concentration of platelet-poor plasma (5%) for 12 hr to determine if plasma can induce cells that have not been treated with PDGF to progress toward the S phase. The medium containing the plasma was removed and replaced with medium containing 50 μg of a heat-treated platelet extract, fresh plasma, and [^{3}H]dThd. The platelet extract and plasma were also added to duplicate cultures that had not been pretreated with plasma, and the entry of cells into the S phase was measured by autoradiography. Both sets of cultures began to synthesize DNA 12 hr after the addition of the platelet extract and platelet-poor plasma (Fig. 1). In addition, the rates of entry into the S phase were identical. Thus, treatment of cells with plasma before the addition of PDGF does not induce the cells to progress toward the S phase. Treatment with PDGF is required for cells to respond to growth factors in platelet-poor plasma.

Latent Period before Cells Progress beyond the Competence Point. Platelet-poor plasma induced PDGF-treated competent BALB/c-3T3 cells to enter the S phase after a lag of 12 hr (Fig. 2A). Growth arrest points in the plasma-dependent progression through G_0/G_1 may occur during this lag period before DNA synthesis. These points might be detected by a shortened (less than 12 hr) interval to DNA synthesis after the addition of an optimal concentration of plasma. BALB/c-3T3 cells were induced to become competent by a 5-hr exposure to a heat-treated platelet extract containing PDGF (4). The competent BALB/c-3T3 cells were then stimulated with

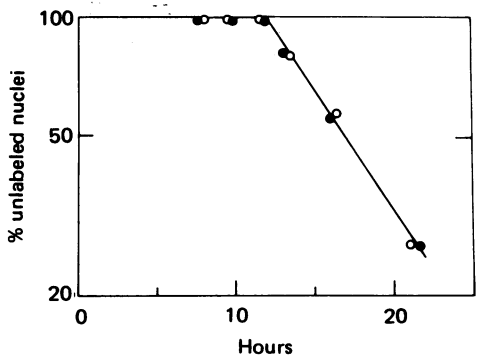

FIG. 1. Pretreatment of G_0-arrested BALB/c-3T3 cells with plasma does not affect entry into the S phase. Platelet-poor plasma (final concentration, 5%) was added (●) or not added (○) to quiescent cultures of BALB/c-3T3 cells. Twelve hours later, the medium (with or without plasma) was removed and replaced with 0.2 ml of medium containing 50 μg of platelet extract, 5% platelet-poor plasma, and [^{3}H]dThd. At the indicated times, the cultures were fixed and processed for autoradiography.

an optimal concentration of plasma for 5 hr followed by transfer to medium lacking plasma. The short exposure to plasma did not stimulate the cells to enter the S phase (Fig. 2*B*). The addition of fresh plasma to duplicate cultures 10 hr after the initial plasma was removed induced the cells to synthesize DNA. They entered the S phase 12 hr after the second addition of plasma. Thus, the initial 5-hr treatment with plasma did not induce these cells to leave the competence point and progress toward the S phase. Similarly, treatment of PDGF-treated competent BALB/c-3T3 cells with limiting concentrations of plasma (0.25–1%) for 12 hr did not induce them to leave the competence point; the subsequent addition of an optimal concentration (5%) of plasma induced these cells to enter the S phase after a lag of 12 hr (Fig. 3 *A* and *B*).

Growth Arrest Point (V) 6 Hr before S Phase. PDGF-treated competent cells were also exposed to 5% plasma for 10 hr. Many of these cells (40%) synthesized DNA, but cell entry into the S phase ceased 5 hr after the initial plasma was withdrawn. A second plasma treatment stimulated those cells that had not become committed to DNA synthesis by the first plasma exposure to synthesize DNA after a lag of only 6 hr (Fig. 2*C*). Thus, a 10-hr treatment of competent BALB/c-3T3 cells with an optimal concentration of plasma stimulates a population of cells to progress through G_0/G_1 and synthesize DNA. Other cells, however, become arrested at a midpoint in G_0/G_1, 6 hr before entry into the S phase. These cells could be stimulated to synthesize DNA by the addition of fresh plasma. For the purpose of discussion, this mid-G_0/G_1 arrest point will be referred to as the V point.

Stimulation of PDGF-treated competent BALB/c-3T3 cells with limiting concentrations of platelet-poor plasma confirmed that cells become arrested 6 hr before the S phase. Treatment with 2, 2.5, or 3% platelet-poor plasma induced 36, 50, or 66% of the cells, respectively, to synthesize DNA (Fig. 3 C–E). Addition of an optimal concentration of platelet-poor plasma after a 12-hr incubation in these limiting plasma concentrations induced a population of cells that had previously not entered the S phase to synthesize DNA after a lag of only 6 hr. Clearly, a group of competent BALB/c-3T3 cells exposed to 2.0–3.0% plasma progressed to a point in the cell cycle that is 6 hr before the S phase but did not progress further. The arrest point for these cultures is probably the same V point that was noted in the plasma withdrawal experiments (Fig. 2*C*).

Growth Arrest Point (W) Immediately before S Phase. To

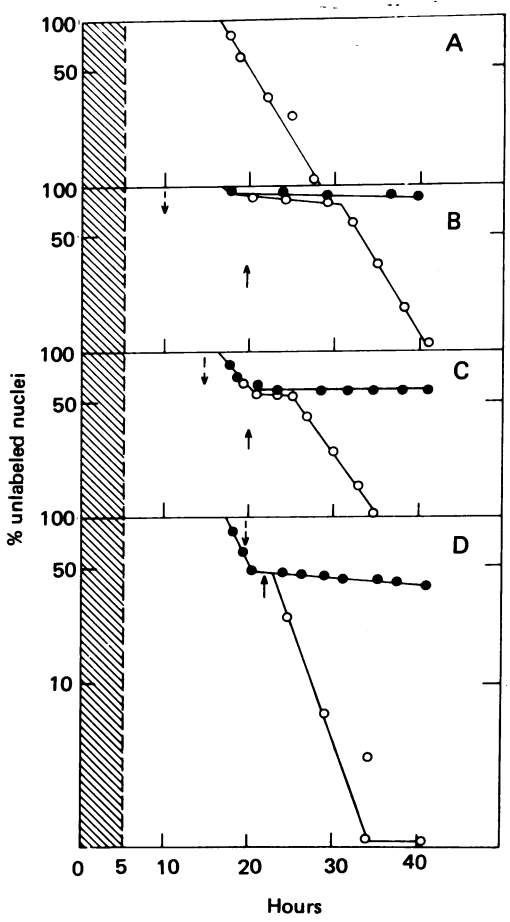

FIG. 2. Sequential growth arrest points in the plasma-dependent progression of BALB/c-3T3 cells through G_0/G_1. Cultures were treated with 50 μg of the platelet extract for 5 hr at 37° (hatched bar) in 0.2 ml of medium. The platelet extract was then removed, and the cells were washed and returned to medium (0.2 ml) containing [^{3}H]-dThd and 5% plasma. (*A*) At the indicated times, cultures were fixed and processed for autoradiography. (*B–D*) At the indicated times (↓), the cultures were transferred to medium (0.2 ml) lacking plasma but containing [^{3}H]dThd [●, no further additions; ○, at the times indicated (↑), the cultures were again transferred to medium (0.2 ml) containing 5% plasma and [^{3}H]dThd]. The cultures were fixed and processed for autoradiography at timed intervals.

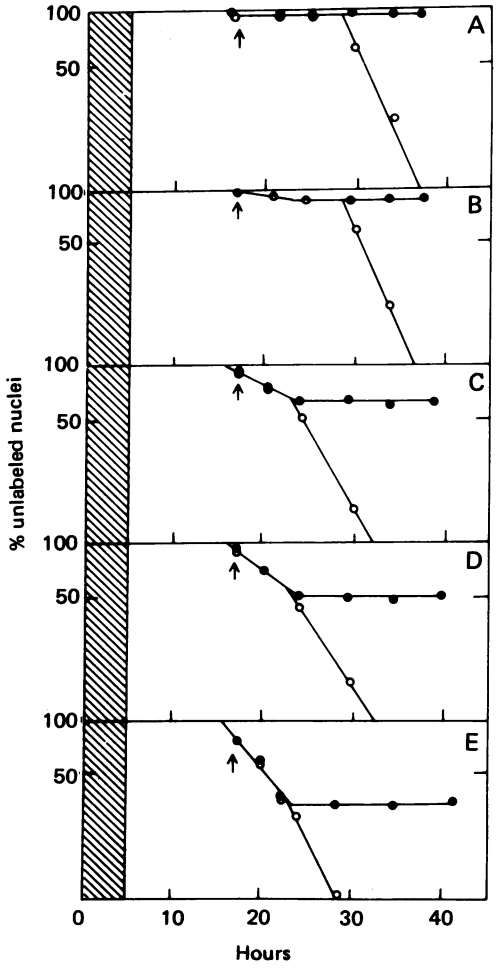

FIG. 3. Competent BALB/c-3T3 cells in medium supplemented with 2–3% plasma become arrested at the V point in G_0/G_1, 6 hr from the S phase. Cultures were treated with 50 μg of the platelet extract (hatched bar) for 5 hr as described in Fig. 2. The platelet extract was removed, and the cultures were washed and returned to medium (0.2 ml) containing [^{3}H]dThd and platelet-poor plasma: (*A*) 0.25%; (*B*) 1.0%; (*C*) 2.0%; (*D*) 2.5%; and (*E*) 3.0%. No further additions were made (●) or, at the time indicated (↑), the medium was removed and replaced with medium (0.2 ml) containing [^{3}H]dThd and 5% plasma (○). The cultures were fixed and processed for autoradiography at timed intervals.

demonstrate that BALB/c-3T3 cells can be arrested after the V point, PDGF-treated competent cells were stimulated with 5% platelet-poor plasma for 15 hr before the plasma was removed. After a 15-hr exposure to plasma, 56% of the cells entered the S phase; however, the remainder of the population became growth arrested within 1–2 hr of plasma withdrawal (Fig. 2*D*). Similarly, chicken embryo fibroblasts treated with multiplication-stimulating activity (MSA) rapidly become growth-arrested when the MSA is removed (14).

An optimal concentration of platelet-poor plasma (5%) was then added to a duplicate culture of growth-arrested BALB/c-3T3 cells 3 hr after the initial plasma was withdrawn. This second plasma addition stimulated the BALB/c-3T3 cells that had previously become growth arrested to begin to synthesize DNA within 1–2 hr. Thus, a population of competent BALB/c-3T3 cells exposed to plasma for 15 hr before being shifted to medium alone appears to become growth arrested just prior to DNA synthesis. Clearly, these cells are arrested at a point in G_1 (termed W) just prior to the commitment to DNA synthesis.

The rate at which cells enter the S phase is a direct function of the serum concentration, demonstrating that serum components alter the transition probability (12, 13). The ability to arrest cells at the W point in G_1, just before commitment, allowed us to demonstrate that plasma directly controls the commitment event. Quiescent BALB/c-3T3 cells were treated with platelet extract and platelet-poor plasma for 12 hr and then shifted to medium alone. This treatment induced 25% of the cells to synthesize DNA. Twenty-four hours after the plasma was withdrawn, fresh platelet-poor plasma was added to the cultures. The cells entered the S phase, with apparent first-order kinetics, after the addition of fresh plasma; both the rate of entry into the S phase and the number of cells that synthesized DNA were a function of the plasma concentration (Fig. 4). With 10% plasma, the cells entered the S phase 40-fold more rapidly than with 0.25% plasma. With 2.5% plasma or less, the cells ceased to enter the S phase after 5 hr, whereas with 5–10% plasma, the cells continued to enter for 15 hr. With 10% plasma, the apparent first-order rate of entry extended over one order of magnitude.

The entry of cells into the S phase during the first 5 hr after plasma addition is shown in Fig. 4 *inset*. Again, the rate of entry

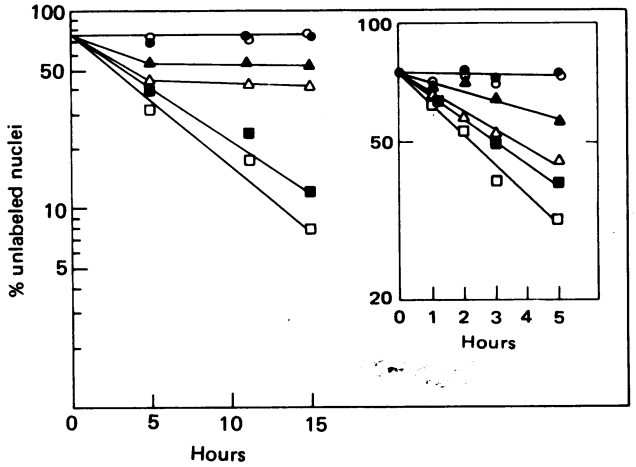

FIG. 4. The entry of BALB/c-3T3 cells into the S phase, from the W point in G_1, is a plasma-dependent event with first-order kinetics and coincides with the time of plasma addition. Quiescent BALB/c-3T3 cells were stimulated with 50 μg of platelet extract for 5 hr as described (Fig. 2) and transferred to medium (0.2 ml) containing [^{3}H]dThd and 5% plasma. Twelve hours later, the medium containing plasma was removed and the cultures were placed in medium (0.2 ml) containing [^{3}H]dThd. After 24 hr, the cultures were placed in medium (0.2 ml) containing [^{3}H]dThd and plasma (○, 0%; ●, 0.25%; ▲, 1%; △, 2.5%; ■, 5%; and □, 10%). At hourly intervals, the cultures were fixed and processed for autoradiography. Cells were fixed during the first 15 hr after plasma addition. (*Inset*) Expanded scale of the culture during the first 5 hr after plasma addition.

was dependent upon the plasma concentration. The cells entered the S phase immediately after plasma addition because the rate of entry curves could be extrapolated back to 0 time (the W point).

This experiment demonstrates that BALB/c-3T3 cells can remain arrested at the W point for as long as 24 hr after plasma withdrawal. These cells are not committed to DNA synthesis because they do not enter the S phase unless plasma is added. Thus, plasma commits W-point arrested cells to enter the S phase. The rate of commitment to DNA synthesis is determined by the concentration of plasma. Because DNA synthesis begins immediately after plasma addition, the actual commitment to DNA synthesis (transition) occurs immediately before the S phase (in G_1) or else occurs as DNA synthesis begins (S phase).

Hydroxyurea Does Not Block Commitment. Hydroxyurea is an inhibitor of DNA synthesis. To show that the commitment to DNA synthesis occurs before a hydroxyurea block of the S phase, cells at the W point were stimulated with an optimal concentration of plasma (5%) in the presence of 1.0 mM hydroxyurea, a concentration sufficient to inhibit replicative DNA synthesis. Twelve hours later, the hydroxyurea was removed and the cells were transferred to medium containing or lacking plasma. Cells released from hydroxyurea block entered the S phase synchronously (rather than with apparent first-order kinetics), both in the presence and in the absence of plasma (Fig. 5*A*). Clearly, the initiation of DNA synthesis after hydroxyurea release is not plasma-dependent. Therefore, the W point and the plasma-dependent commitment to DNA synthesis are before the hydroxyurea block of the S phase.

Hydroxyurea inhibits the enzyme ribonucleotide reductase (15). In the presence of hydroxyurea, DNA synthesis stops within a short time because cells deplete their pool of deoxyribonucleotides. However, hydroxyurea does not prevent the actual initiation of DNA synthesis, which occurs before pool depletion (16). Thus, the exit from W and the transition to the committed state may represent the actual initiation of DNA synthesis.

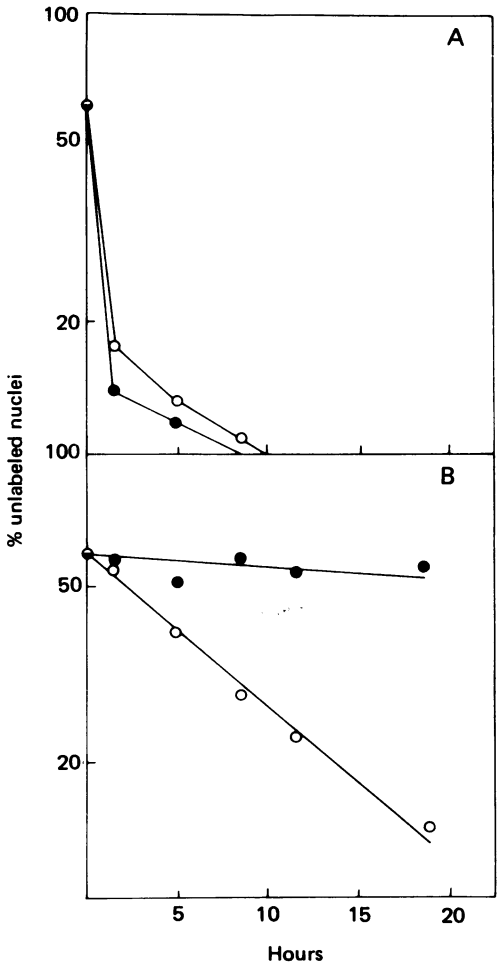

FIG. 5. The transition to the committed state is blocked by cycloheximide but not by hydroxyurea. Quiescent BALB/c-3T3 cells were stimulated with 50 μg of platelet extract for 5 hr, washed, and transferred to medium (0.2 ml) containing 5% plasma and [^{3}H]dThd for 15 hr. The cultures were then transferred to medium (0.2 ml) containing [^{3}H]dThd but no plasma for 24 hr as described in Fig. 4. The medium was replaced with medium (0.2 ml) containing 5% plasma, [^{3}H]dThd, and 1.0 mM hydroxyurea (*A*) or cycloheximide at 7.5 μg/ml (*B*). After 12 hr, the cultures were washed and transferred to fresh medium (0.2 ml) containing [^{3}H]dThd with (○) or without (●) plasma (5%). The cells were fixed and processed at intervals for autoradiography.

Commitment (Transition) is Blocked by Cycloheximide. Brooks (17) has found that entry of cells into the S phase is rapidly inhibited by cycloheximide. To demonstrate directly that the plasma-regulated commitment event is blocked by cycloheximide, cells arrested at the W point were stimulated with an optimal concentration of plasma in the presence of cycloheximide at 7.5 μg/ml, a concentration sufficient to inhibit greater than 95% of protein synthesis. Twelve hours later, the cycloheximide was removed, the cultures were transferred to medium with or without plasma, and the entry of cells into the S phase was determined. Cells treated with plasma in the presence of cycloheximide did not enter the S phase when they were transferred to medium alone (Fig. 5*B*). These cycloheximide-treated cells remained arrested at the W point because they rapidly entered the S phase when they were shifted to medium containing plasma. In order to become committed to DNA synthesis, cells must synthesize protein while they are

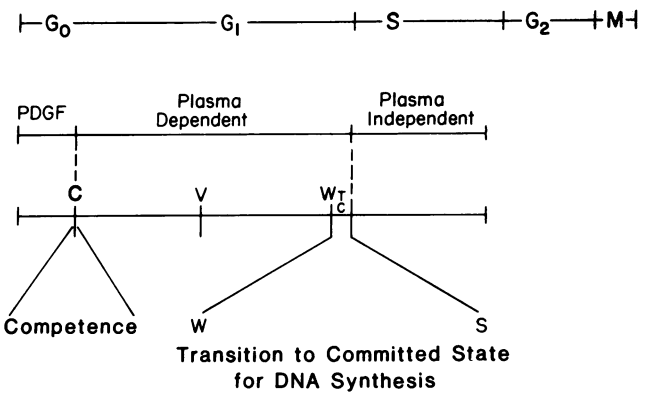

FIG. 6. Model of several sequential events in G_0/G_1 that precede DNA synthesis.

being treated with plasma. Cells that cannot synthesize protein, or cells that are not exposed to plasma, remain arrested at the W point.

DISCUSSION

Serum induces BALB/c-3T3 cells to proliferate by controlling an ordered sequence of events directed toward replication in the G_0/G_1 phase of the cell cycle. These ordered events have been recognized by the sequential addition of serum components (PDGF and platelet-poor plasma) to density-inhibited cells. Removal of these components at timed intervals allowed the recognition of distinct growth arrest points in G_0/G_1. PDGF induces density-inhibited cells to reach the first growth arrest point, the competence point (4). Cells remain at the competence point for as long as 13 hr after PDGF is removed (4). A prolonged exposure to platelet-poor plasma induces these competent cells to enter the S phase. The first growth arrest point in the plasma-dependent sequence, the V point, occurs 6 hr before S phase; the second, the W point, occurs immediately before DNA synthesis. W-point arrested cells are not committed to DNA synthesis. The actual commitment (transition) event occurs as cells leave the W point and enter the S phase. This ordered sequence of events is regulated by platelet-poor plasma and appears to require protein synthesis. This ordered sequence of replicative events is depicted in Fig. 6.

The recognition of the competence, V, and W points demonstrates that an ordered sequence of events directed toward cellular replication occurs during G_0/G_1. Other growth arrest points may also exist in G_0/G_1. Because plasma-mediated progression of PDGF-treated competent cells into S phase is a function of both the plasma concentration and the length of exposure, these growth arrest points might be recognized by varying these two factors. Other methods of inducing growth arrest, such as amino acid deprivation, may also prove useful in defining sequential growth arrest points.

Baserga (18) has proposed that "gene activation" causes a cascade of biochemical events (including RNA and protein synthesis) which leads to DNA synthesis. In serum-stimulated 3T3 cells, RNA synthesis occurs before DNA synthesis (19). The present data demonstrate a series of sequential growth arrest points before commitment occurs, but biochemical analysis of the cell cycle was not undertaken.

Pardee (20) has proposed that a growth arrest point, termed the "restriction point," controls the entry of G_0-arrested cells into the proliferative phase of the cell cycle. The growth of viral-transformed cells is not regulated by this growth arrest point (20). The present data demonstrate the presence of additional growth arrest points in the G_0/G_1 phase of the BALB/c-3T3 cell cycle.

The model that we propose differs from the transition probability view (10, 11) of the cell cycle in one important way. According to the transition probability model, the commitment of cells to replicate is described solely by a single random event that is rate-limiting (the transition probability). It was suggested that the events that occur during the A state are not directed toward cellular replication (10, 11). The present data demonstrate that a transition event that occurs as cells enter the S phase has an important role in regulating the commitment of cells to synthesize DNA. However, this transition is preceded by an ordered sequence of events, defined by a series of growth arrest points. Serum growth factors, derived from platelets and plasma, regulate the ordered cell cycle events that precede commitment and also regulate the commitment event itself. Because these serum factors control several distinct events in G_0/G_1, cells can spend an indeterminant amount of time in this phase of the cell cycle. The ordered events that occur after commitment are serum independent (unpublished data) and thus appear to be regulated intrinsically by the cell itself. The data support a sequential model of the cell cycle (Fig. 6). The transition probability describes only one step in an ordered series of replicative events that occurs during G_0/G_1.

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