ABSTRACT  This paper explores the transitional states that bridge the gap between nuclear quiescence and mitogenesis. It presents evidence for the formation of a committed but prereplicative state. Quiescent murine Swiss 3T3 cells were exposed to an external mitogenic stimulant (epidermal growth factor or excess serum) and simultaneously to a synchronizer which inhibits entry into the S phase. Thus, the cells were stimulated to synthesize DNA, but the normal replicative response to this stimulus was blocked. The block to DNA replication was removed at varying times after removal of the stimulant. Experiments were done to monitor the decay of commitment to DNA synthesis after removal of the external stimulant. This decay turned out to be a first-order process. The half-life (time required for loss of the commitment to DNA synthesis in half of the initially sensitized cells) was found to be approximately 5 hr. The same result was found whether total DNA synthesis or individually replicating cells were measured and was independent of the type of external growth stimulant or blocker used. These results point to the existence, on the mitogenic pathway, of a committed but prereplicative state. The committed state appears to represent a unit or global property of the whole cell rather than, for example, a critical concentration of some active inducer molecule.

MATERIALS AND METHODS

Materials. Hydroxyurea and unlabeled thymidine were from Sigma. [3H]Thymidine (20 Ci/mmol; 1 Ci = 3.7 × 10^10 becquerels) was a product of New England Nuclear. Calf serum (fetal and newborn) was from GIBCO. Murine epidermal growth factor (EGF) was purified as described (16). Swiss mouse 3T3 cells were obtained from American Type Culture Collection.

Cell Culture. Monolayer cultures of homogeneous Swiss mouse 3T3 cells were grown and maintained at 37°C in a 10% CO₂/90% air atmosphere in Dulbecco's modified Eagle's medium (DME medium) containing 3% fetal calf serum and 7% newborn calf serum. Cells were subcultured by trypsinization. Routine tests showed the cells to be free of mycoplasma contamination.

DNA Synthesis. Incorporation of [3H]thymidine into DNA by cell monolayers was determined as described (8). Cell monolayers in 16-mm dishes were incubated at 37°C for 1 hr with [3H]thymidine (1 μCi/ml; 1.5 Ci/mmol) in 0.5 ml of DME medium containing 2% fetal calf serum. At the end of incubation, the cell monolayers were washed twice with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4, and incubated at 4°C for 20 min with 1 ml of 5% trichloroacetic acid. The acid-insoluble radioactivity sticking to the culture dishes was washed once with 5% trichloroacetic acid and twice with methanol. The insoluble material was then dissolved with 0.5 M NaOH, neutralized with HCl, and assayed for radioactivity in a xylene-based scintillation fluid.

Nuclear Labeling by Autoradiography. For determination of nuclear labeling index (17), cells grown on glass coverslips were incubated with [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) as described in the legends for Figs. 3 and 4. At the end of incubation, the monolayers were washed twice with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4, and fixed in methanol at 4°C for 5 min. The coverslips were air-dried, mounted on glass slides, and then treated with NTB-2 emulsion (Kodak). After a 24-hr incubation in the dark, the slides were developed and fixed. Labeled nuclei were visualized as spots containing at least five dark grains. The unlabeled nuclei were stained with hematoxylin (Fisher). For each determination of percentage labeled nuclei, at least 400 cells were counted. Increasing the development time from 24 hr to 48 hr did not significantly increase the nuclear labeling index under these experimental conditions.

Abbreviations: EGF, epidermal growth factor; DME medium, Dulbecco's modified Eagle's medium.

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RESULTS

For determining the rate of inactivation of the induced DNA synthetic ability, the quiescent cells were exposed for 15 hr to both a mitogenic stimulant (excess serum or a mitogenic hormone) and a synchronizer which caused arrest at the G1/S interface (hydroxyurea or excess thymidine). Thus, the cells were stimulated to become committed to synthesize DNA, but the replicative expression of this commitment was blocked. The block to DNA replication was removed at varying times after removal of the stimulant. These experiments, therefore, afforded a means for determining the decay of commitment to synthesize DNA after removal of the external stimulant.

Fig. 1A depicts DNA replication induced by EGF in mouse 3T3 cells. In these experiments hydroxyurea (18) was used as the cell-synchronizing agent. After the removal of hydroxyurea, it took about 6 hr for the peak of DNA synthesis to appear. DNA synthetic activity, measured by the peak height or area, decreased with increasing time of blockage after EGF removal. A plot of DNA synthetic activity at peak hours (incorporation at 4 plus 8 hr after removal of hydroxyurea) against time after EGF removal revealed that the decay was a first-order process (Fig. 2). The time required for loss of the commitment to DNA synthesis in half of the initially sensitized cells (subsequently referred to as half-life) was approximately 5 hr.

In order to determine whether hydroxyurea per se had any gross influence upon the decay of induced DNA synthetic activity, other half-life determinations were done using excess thymidine (19) as the cell-synchronizing agent and either EGF or serum as the external stimulant. In the EGF/thymidine experiments, DNA synthesis peaked at about 3–6 hr after removal of the excess thymidine (Fig. 1B). Here also, decay was found to be a first-order process, and the half-life was the same as in the EGF/hydroxyurea experiments (Fig. 2). In the serum/thymidine experiments, the time required for inactivation of the serum-induced committed state in half of the sensitized cells was also about 5 hr. This suggests that the in vivo decay of induced commitment is not influenced by these inhibitors. The alternative, that the decay is affected to the same extent by both hydroxyurea and thymidine, seems less likely.

The constancy of the half-time value (Fig. 2) also suggests that the nature of the external inducing agent has no influence upon the stability of the internal committed state and that the same internal state is generated in response to both serum and EGF.

In a control experiment with excess thymidine in which the cells were not treated with any external stimulant, the absence of DNA synthetic peaks clearly confirms the absence of nuclear replicative activity in a large majority of these unstimulated cells (Fig. 1D).

The time-dependent decay of DNA synthetic activity shown in Figs. 1 and 2 can be interpreted to represent either a gradual

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The cells for 20 hr in the serum. Cells (B) at cells and Quiescent cultures then hr but incubated different densities, cultures of 4. FIG. 37°C for 15% fetal calf serum. The end of incubation, cells were washed with conditioned medium and then incubated at 37°C in conditioned medium containing 2 mM hydroxyurea but no additional serum for various periods (0, 3, 6, 9, 12, and 15 hr (c); 0, 4, 8, and 12 hr (d)). The hydroxyurea-containing medium was then removed, and conditioned medium (without hydroxyurea or additional serum) containing [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) was added to each dish. After a 12-hr incubation at 37°C, percentage labeled nuclei were determined.

**FIG. 3.** Nuclear autoradiographic determination of decay. Quiescent cultures of 3T3 cells in glass cover slips were maintained at two different densities, 2 × 10^4 cells per cm^2 (c) and 5 × 10^4 cells per cm^2 (d). These cells were incubated at 37°C for 15 hr with 2 mM hydroxyurea in conditioned medium (DME medium containing 2% fetal calf serum) containing additional 15% fresh fetal calf serum. At the end of incubation, cells were washed with conditioned medium and then incubated at 37°C in conditioned medium containing 2 mM hydroxyurea but no additional serum for various periods (0, 3, 6, 9, 12, and 15 hr (c); 0, 4, 8, and 12 hr (d)). The hydroxyurea-containing medium was then removed, and conditioned medium (without hydroxyurea or additional serum) containing [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) was added to each dish. After a 12-hr incubation at 37°C, percentage labeled nuclei were determined.

**FIG. 4.** Decay of commitment and restimulation with serum. Quiescent cultures of 3T3 cells on glass cover slips were incubated at 37°C for 15 hr with 15% fetal calf serum in conditioned medium (DME medium containing 2% fetal calf serum) containing 2 mM hydroxyurea. Then, the cells were treated as follows, and percentage labeled nuclei was determined. (A) Cells were washed with conditioned medium, and nuclear stimulation was determined by incubating the cells at 37°C for 12 hr with [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) in conditioned medium containing no hydroxyurea or added serum. (B) Cells were washed with conditioned medium, and the stimulation was allowed to decay by incubating the cells at 37°C for 15 hr with 2 mM hydroxyurea in conditioned medium containing no additional serum. At the end of incubation, the decay was determined by incubating the cells at 37°C for 12 hr with [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) in conditioned medium containing no hydroxyurea or additional serum. (C) The cells were washed with conditioned medium, and the stimulation was allowed to decay by incubating the cells as in B. The cells were then tested for restimulation by incubation at 37°C for 20 hr with 15% fresh fetal calf serum and [3H]thymidine (0.5 μCi/ml; 1.5 Ci/mmol) in conditioned medium containing no hydroxyurea.

**FIG. 5.** Effect of prolonged exposure to hydroxyurea and thymidine upon nuclear stimulatory response to serum. Quiescent cultures of 3T3 cells in 16-mm dishes were incubated at 37°C for various periods (15-30 hr) with 15% fetal calf serum in conditioned medium (DME medium containing 2% fetal calf serum) containing either 2 mM hydroxyurea or 7.5 mM thymidine. At the end of incubation the cells were washed with conditioned medium, and [3H]thymidine incorporation into DNA was measured at regular time intervals as in Fig. 1. Incorporations at 4 and 8 hr after removal of hydroxyurea/serum were combined in each case and plotted against time of exposure to hydroxyurea/serum (c). In the thymidine/serum experiments (d), incorporations at 3 and 6 hr after removal of thymidine/serum were combined in each case and plotted against time of exposure to thymidine/serum. ×, No treatment.

The data shown in Figs. 1-3 were reproducible and representative of a large number of decay experiments in which first-order decay kinetics (with a half-life of 5 hr) were consistently observed. This close correspondence between data from separate experiments suggests that these kinetic observations are probably true and unlikely to be the results of errors or artifacts. The decrease in DNA synthetic ability with increasing time after stimulant removal showed the characteristics of a first-order decay, and the half-life was again found to be 5 hr. The decay rate was the same irrespective of the initial degree of a nuclear stimulation and was independent of the development time (24-48 hr) used for autoradiography.

The following points are worth noting. (a) The cells used in these experiments were synchronously quiescent and density-inhibited (Fig. 1D). (b) The decay of induced DNA synthetic activity was exponential over 15 hr, corresponding to three half-lives.

**DISCUSSION**

The data shown in Figs. 1-3 were reproducible and representative of a large number of decay experiments in which first-order decay kinetics (with a half-life of 5 hr) were consistently observed. This close correspondence between data from separate experiments suggests that these kinetic observations are probably true and unlikely to be the results of errors or artifacts. The decrease in DNA synthetic ability with increasing time after stimulant removal showed the characteristics of a first-order decay, and the half-life was again found to be 5 hr. The decay rate was the same irrespective of the initial degree of a nuclear stimulation and was independent of the development time (24-48 hr) used for autoradiography.

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In these calculations, it is assumed that the molecular concentration shows a Gaussian distribution (broad \( \sigma = C_{\text{cont}} \), or narrow \( \sigma = C_{\text{cont}} \)) within a cell population (Inset), but the general results will hold for any reasonable concentration distribution. In the hypothetical case depicted here, there is 93% commitment at time 0 (i.e., 93% of the cells contain a greater than critical concentration \( C_{\text{cont}} \) of the active inducer molecule). Thus, \( C_{\text{cont}} \) is the concentration at 1.5 \( \sigma \) (standard deviations) below the mean. With passage of time, in the absence of further stimulation, the molecular concentrations decay, causing a sliding of the Gaussian profile so that cells transit from \( >C_{\text{cont}} \) to \( <C_{\text{cont}} \). The time taken for this loss of commitment (\( >C_{\text{cont}} \rightarrow <C_{\text{cont}} \) transition) for cells having an initial level of \( C_0 \) is proportional to \( \log \left( C_0/C_{\text{cont}} \right) \). If a rather broad concentration distribution is assumed (0.5 \( \sigma = C_{\text{cont}} \)), relative times for the decays 93% \( \rightarrow \) 84%, 93% \( \rightarrow \) 69%, 93% \( \rightarrow \) 50%, 93% \( \rightarrow \) 31%, and 93% \( \rightarrow \) 16% are 1.5, 2.4, 3, 3.5, and 3.9 time units, respectively (unbroken line). If a very narrow distribution is assumed (0.5 \( \sigma = C_{\text{cont}} \), relative times for the decays 93% \( \rightarrow \) 84%, 93% \( \rightarrow \) 69%, 93% \( \rightarrow \) 50%, 93% \( \rightarrow \) 31%, and 93% \( \rightarrow \) 16% are 0.75, 1.5, 2.25, 3, and 3.75 time units, respectively (broken line).

(i.e., it showed the characteristics of a first-order reaction) (Figs. 2 and 3). (c) The observed decay of induced DNA synthetic activity was not due to loss of cell viability. (d) The time-dependent decay of induced DNA synthetic activity represented a quantal decrease in the number of cells replicating their genome rather than a uniform continuous decrease in DNA synthetic rate in all the cells (Fig. 3). (e) The cell-synchronizing agents used for decay determinations had no irreversible inhibitory effect upon the mitogenic potential of 3T3 cells (Figs. 4 and 5). Cells in which commitment had decayed could be restimulated by addition of fresh external stimulant (Fig. 4). (f) Internal commitment produced with either EGF or serum (platelet factor is the principal mitogen in serum) had identical half-lives and the half-life was the same irrespective of the type of synchronizer used. These suggest the absence of specific effects of either hydroxyurea or thymidine upon the decay characteristics of the committed state and also suggest the production of a common internal state in response to diverse stimuli. These data have led to the formulation of a model for commitment, which is discussed in detail below.

Regarding the internal process by which resting cells become committed to synthesize DNA, one can consider at least two different models: (i) commitment to synthesize DNA represents the build-up of a critical concentration of a labile initiator molecule; or (ii) commitment represents a distinctive state within the cell, perhaps coded into the configuration of some cellular macrostructure such as nuclear membrane or a part of the genome, which might require surpassing a critical concentration of active molecules earlier in the pathway, but the state itself represents a yes/no whole-cell decision. Normally, attainment of \( C^* \) state would lead to DNA replication and subsequent cell division; but, in the presence of inhibitors (hydroxyurea/excess thymidine), DNA replication is blocked and the state decays. ESI, external stimulant-induced commitment pathway.

The results reported in this paper suggest that the first model is unlikely. This model predicts that, under normal conditions of stimulation—i.e., in the absence of hydroxyurea/excess thymidine or other replication blocking agents—DNA replication would proceed as soon as the cell achieves the critical concentration of the active inducer molecule. In the presence of a replication blocking agent, the active molecule would continue to accumulate due to persistent external stimulation but would be ineffective. If one visualizes a Gaussian distribution for this molecule within a stimulated (but blocked) cell population (Fig. 6/Inset), then the decay of commitment to synthesize DNA will exhibit a lag phase characteristic of multi-hit kinetics, a delay period during which the concentration in various cells sinks toward the critical level, and then an accelerating loss of commitment as more and more cells go below the critical concentration (Fig. 6). In actuality, such a lag was never observed, and the decay turned out to be a first-order process. This suggests a plus-minus or on/off type of mechanism for control of DNA replication.

According to model b (Fig. 7), the committed replication-inducing state (\( C^* \) state) is present only in a single copy per cell, and it decays in a single-step reaction. All \( C^* \) states in a cell population have the same probability for decay, independent of their past history of induction and the life of any \( C^* \) state in a cell population—i.e., the length of time it can exist before it decays can have all possible values from zero to infinity. Thus, this kind of model can explain the experimentally observed exponential decay of commitment. The half-life is an important

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**Fig. 6.** Theoretical decay curves for the molecular concentration model. In these calculations, it is assumed that the molecular concentration shows a Gaussian distribution (broad \(-\rightarrow\), 0.5 \( \sigma = C_{\text{cont}} \), or narrow \(-\rightarrow\), 0.5 \( \sigma = C_{\text{cont}} \)) within a cell population (Inset), but the general results will hold for any reasonable concentration distribution. The hypothetical case depicted here, there is 93% commitment at time 0 (i.e., 93% of the cells contain a greater than critical concentration \( C_{\text{cont}} \) of the active inducer molecule). Thus, \( C_{\text{cont}} \) is the concentration at 1.5 \( \sigma \) (standard deviations) below the mean. With passage of time, in the absence of further stimulation, the molecular concentrations decay, causing a sliding of the Gaussian profile so that cells transit from \( >C_{\text{cont}} \) to \( <C_{\text{cont}} \). The time taken for this loss of commitment (\( >C_{\text{cont}} \rightarrow <C_{\text{cont}} \) transition) for cells having an initial level of \( C_0 \) is proportional to \( \log \left( C_0/C_{\text{cont}} \right) \). If a rather broad concentration distribution is assumed (0.5 \( \sigma = C_{\text{cont}} \)), relative times for the decays 93% \( \rightarrow \) 84%, 93% \( \rightarrow \) 69%, 93% \( \rightarrow \) 50%, 93% \( \rightarrow \) 31%, and 93% \( \rightarrow \) 16% are 1.5, 2.4, 3, 3.5, and 3.9 time units, respectively (unbroken line). If a very narrow distribution is assumed (0.5 \( \sigma = C_{\text{cont}} \), relative times for the decays 93% \( \rightarrow \) 84%, 93% \( \rightarrow \) 69%, 93% \( \rightarrow \) 50%, 93% \( \rightarrow \) 31%, and 93% \( \rightarrow \) 16% are 0.75, 1.5, 2.25, 3, and 3.75 time units, respectively (broken line).

![Diagram](image_url)

**Fig. 7.** Committed (\( C^* \)) state model. The ultimate committed (\( C^* \)) state appears to be a unit or global property of the whole cell rather than, for example, a critical concentration of some active inducer molecule. The committed state is perhaps coded into the configuration of some cellular macrostructure such as nuclear membrane or a part of the genome, which might require surpassing a critical concentration of active molecules earlier in the pathway, but the state itself represents a yes/no whole-cell decision. Normally, attainment of \( C^* \) state would lead to DNA replication and subsequent cell division; but, in the presence of inhibitors (hydroxyurea/excess thymidine), DNA replication is blocked and the state decays. ESI, external stimulant-induced commitment pathway.
quantity in exponential decay because it gives the time that must elapse for the activity to decay to half its value. This value is independent of the initial activity or the initial number of cells containing the committed state. As experimentally observed, this value is a constant, independent of the initial degree of stimulation. It appears to be an innate, inherent property of the cell type, independent of the external condition used for achieving commitment.

Thus, the results reported here point to the existence on the mitogenic pathway of a committed but prereplicative state (Fig. 7). Left to itself under normal conditions, the C* state would lead to a complete round of DNA replication accompanied perhaps by its own dissolution; but in the presence of inhibitors of DNA synthesis (hydroxyurea or excess thymidine), the state decays, resulting in a transition from nuclear commitment to quiescence. The decay properties of this state may be important determining factors in controlling the length of the G1 phase, and it is of interest to determine this decay in various animal cells under different conditions of stimulation.

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