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Cell Biology

Resting state in normal and simian virus 40 transformed Chinese hamster lung cells*

(cell cycle/simian virus 40 temperature-sensitive transformation/cell synchrony)

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ABSTRACT **Normal cells deprived of amino acids or serum factors enter a resting state, whereas cells transformed by wild-type simian virus 40 do not. The ability to enter a resting state is temperature-sensitive (*ts*) in cells transformed by a *tsA* mutant of simian virus 40. We show further: (*i*) that when complete medium is added to resting cells, the length of time until the onset of DNA synthesis often exceeds the length of G_1 in growing cells; (*ii*) that the length of this interval depends upon the conditions used to arrest cell growth; but (*iii*) that transferring cultures from medium depleted for one factor to medium depleted in a second factor never leads to a round of DNA synthesis; and (*iv*) that DNA synthesis does not resume rapidly when a resting culture of cells transformed by the *tsA* mutant is transferred to the permissive temperature in suboptimal medium. A model proposing that in suboptimal conditions cells leave the cell cycle and traverse a branch pathway to enter the resting state is consistent with these findings.**

The *A* gene of simian virus 40 (SV40) that encodes the "T-antigen" (1, 2) is required for the initiation of viral DNA synthesis and the induction of host DNA synthesis in permissive cells (3–5), and for the maintenance of transformation in nonpermissive cells (6–10). It has therefore been proposed that transformation is a direct consequence of the aberrant initiation of host DNA synthesis by the *A* gene product (11, 12). This view has been criticized. (*i*) "The critical difference between transformed and untransformed cells is that the untransformed cells go into a resting state when conditions are not optimal for their growth. Transformed cells continue to grow in such conditions. Transformation is thus an abrogation of the resting state . . . (*ii*) . . . The point in the cell cycle at which cells rest is somewhere in G_1 but not at the border of the *S* phase . . . (*iii*) . . . Therefore, it would appear that transformation is not a process involving the control of DNA synthesis" (13). The data in this paper support assertion (*i*), but cast doubt on assertion (*ii*), and hence on the conclusion (*iii*).

A considerable body of literature (e.g., refs. 14–16) suggests that cells enter a resting state when the culture medium becomes depleted either by the intentional removal of a nutrient or at high cell densities because the growing cells consume some growth factor (17). The resting state, G_0, has been localized to that portion of the cell cycle in which the cell contains an unreplicated genome (14–16). However, considerable controversy exists over the precise location of G_0. Some authors have proposed that resting states exist throughout the G_1 phase of growth, "early-, mid-, or late-G_1" blocks (18). Others (14, 19) have proposed that G_0 lies outside the cell cycle, but have not specified how such a state is reached. Pardee (20) has proposed that the commitment to enter the resting state is made at a single point in mid-G_1, the restriction point (*R*). However, in distinction to others, he finds no significant differences from one depletion condition to another in the time interval from restoration of complete medium to the onset of DNA synthesis. Still others argue that the entire concept of a cell cycle is misleading, and that soon after completing mitosis cells enter a resting state, G_0 (21) or the "*A* state" (22), from which they emerge with a half-life related to the nutritional state of the medium.

Data are presented confirming the experimental bases for many of these disparate views of the resting state. We discuss a model consistent with these data and with the notion that transformation by SV40 is a consequence of the T-antigen acting exclusively as an initiator of DNA synthesis.

MATERIALS AND METHODS

Cells and Media. The normal Chinese hamster lung cells (CHL), *tsA239* transformed CHL cells (*CHLA239L1*), and wild-type transformed CHL cells (*CHLSV15*) have been described (6, 11, 15). Dulbecco-Vogt modified Eagle's medium (National Institutes of Health media supply) was supplemented with penicillin and streptomycin (6), 5×10^{-6} M thymidine [and where indicated with 1 μCi/ml of [^{3}H]thymidine (New England Nuclear, Boston, Mass.)], and 10% fetal bovine serum (Flow Laboratories, Rockville, Md.) or 0.5% serum. Medium deficient in glutamine or isoleucine was supplemented with 2.5% serum. Cultures were incubated under 6% CO_2 at 33° and 8% CO_2 at 40°.

Growth Curves and Analysis of DNA Synthesis. Cultures of subconfluent randomly growing cells at 33° or after passaging for 2 weeks at 40° were treated with trypsin and dispersed in Dulbecco-Vogt medium supplemented with 10% fetal bovine serum, and aliquots of 1 ml were dispensed into 2-cm^2, 24-well plates (Linbro Chemical Company, New Haven, Conn.). After 1 day the medium was changed as indicated. Care was taken to maintain the temperature and CO_2 equilibration of the medium, and no plate was removed from the incubator for more than 10 min. Where indicated, radioactive medium was removed from the samples and the cells were lysed with 0.5 ml of 0.2 M NaOH, 10 mM EDTA, and 0.6% sodium dodecyl sulfate. The lysates were removed, the wells were rinsed with 1 ml of water, and the lysates and rinses were combined. Approximately 50 μg of calf-thymus DNA was added and each sample was precipitated by the addition of 1 ml of cold 20% trichloroacetic acid. The precipitates were collected on filters and washed with four, 5-ml volumes of cold 5% trichloroacetic acid and one, 5-ml volume of 95% ethanol. Filters from duplicate samples were dried, radioactivity was measured, and values were averaged.

Theoretical Analysis. Randomly growing cells double in a generation time *g*. The number of cells at time *t*, (n_t), is given

Abbreviations: *ts*, temperature sensitive; CHL cells, Chinese hamster lung cells; SV40, simian virus 40.

* This article is dedicated to the late Dr. Gordon Tomkins, who first suggested to us that the resting state should be considered as a branch pathway of the cell cycle.

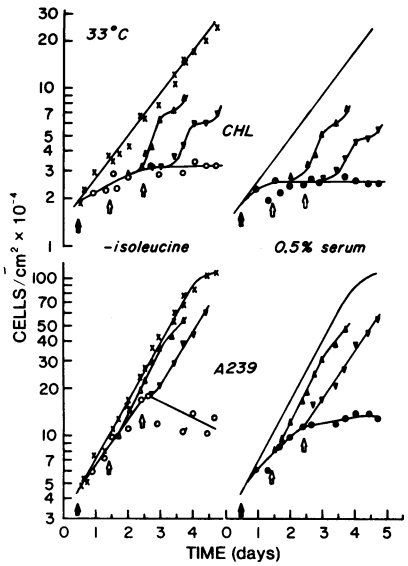

FIG. 1. Growth of normal CHL and SV40 mutant *A239* transformed CHL cells at 33°. The "0" time is arbitrarily set at approximately 12 hr after the cells were pipetted into the wells. At 0.5 day (indicated by the solid arrows) the medium was replaced with medium supplemented with 10% fetal bovine serum (×) or with isoleucine-depleted (○) or 0.5% serum medium (●). The depleted media were replaced with complete medium either 23 or 47 hr later (open arrows) and the increase in cell number was followed (△, ▲ medium replenished at 23 hr; ▽, ▼ medium replenished at 47 hr).

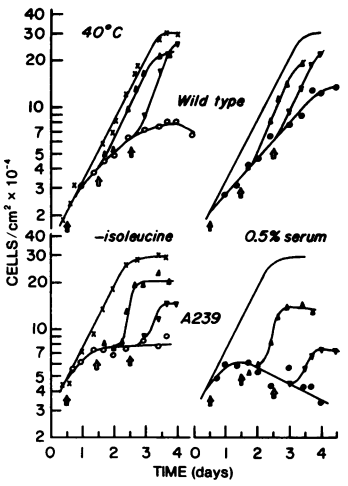

FIG. 2. Growth of SV40 wild-type transformed and *A239* transformed CHL cells at 40°. Conditions and symbols are the same as in Fig. 1 except that the cells were incubated at 40°.

by the equation: $n_t = n_0 2^{t/g}$, where n_0 is the number of cells at "0" time. For a large population of randomly growing cells, DNA synthesis occurs uniformly in time. The radioactivity incorporated into such cells at time t after the addition of radioactive thymidine should be given by a constant, k, times the increase in cell number: $\text{cpm}_t = k(n_t - n_0)$, or $\text{cpm}_t = n_0 k(2^{t/g} - 1)$. The data in Fig. 3 were therefore treated as follows: n and g were determined from Figs. 1, 2, and 6 or from similar data not presented. A value for k was determined by curve fitting to the data when radioactive thymidine was added at 1.5 days. All other curves were then calculated from these values of k and the experimental values of g and n at the time of addition of the radioactive thymidine.

RESULTS

Amino acid or serum depletion results in the synchrony of normal, but not of transformed cells, when assayed by cell number. Randomly growing CHL cells increase in number in complete medium with $g = 26$ hr at 33° (Fig. 1). When a culture is shifted to deficient medium, cell number continues increasing for less than a generation. If complete medium is restored, cell number increases in a step-like manner after a lag of approximately 1 day. Cells transformed by *tsA239*, or by wild-type SV40 (data not shown), behave quite differently. These cells continue growing in deficient medium at 33° but with an increased g, and eventually begin to shed from the monolayer (23), accounting for the apparent plateau seen in the lowest curves in Fig. 1. If complete medium is restored to the starved cells before shedding becomes excessive, cell number increases almost immediately and without any indication of synchrony.

Cells transformed by *tsA239* behave like normal cells when grown at 40° (Fig. 2). However, the *tsA239* transformed cells in complete medium have $g = 18$ hr (normal cells have $g = 32$ hr) and appear to die somewhat more rapidly in 0.5% serum than do normal cells. Nonetheless, the cells transformed by *tsA239* do appear to be synchronized at 40° but not at 33°.

To assess further cell synchrony and to estimate the onset of DNA synthesis, we determined the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material. [^{3}H]Thymidine was added to randomly growing cells at the time corresponding to 1.5 days in Fig. 1 or 0.5 day in Fig. 2. Samples were taken thereafter and the incorporation of thymidine was determined. The data were then fitted to curves having the theoretical shape (see *Materials and Methods*), and a constant, k, was determined for each cell type (Fig. 3). To

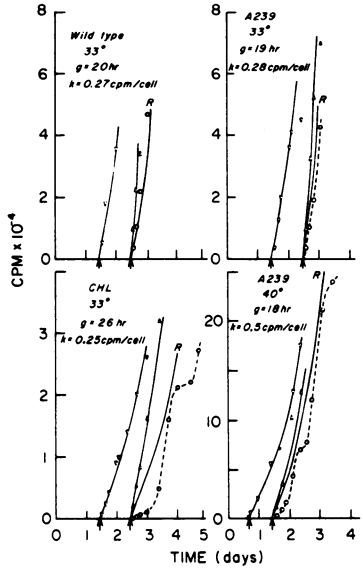

FIG. 3. Thymidine incorporation in cells growing in complete medium or after isoleucine depletion. The treatment of cells is the same as in Figs. 1 and 2 except that at the times indicated by the arrows complete radioactive medium was added. Randomly growing cells were changed to radioactive medium (▽) and the experimental data fitted to the theoretical curve to obtain a value for k (see *Materials and Methods*). Another set of randomly growing cells were changed to radioactive medium 24 hr later (△), and theoretical curves were calculated from the values of g and n determined in Figs. 1 and 2 and the k determined above. At the later time, radioactive medium was also added to cells deprived of isoleucine (○), and theoretical curves for randomly growing cells (solid line, R) were calculated. The experimental results are indicated by the dashed lines.

Table 1. Approximate lag times from the addition of complete medium after deprivation to the onset of *S* phase

	Time to onset of DNA synthesis (hr) after deprivation of					
	Glutamine		Isoleucine		Serum	
Incubation conditions	CHL	*A239*	CHL	*A239*	CHL	*A239*
33°, 24 hr*	8	0	4	0	12	0
33°, 48 hr*	12	0	18	0	20	0
40°, 24 hr*	10	4	11	6	13	8
40°, 48 hr*	11		13	9	14	10
44° → 33°, 24 hr†			18	11	24	10
40° → 33°, 48 hr†			24	14	24	20
24 hr shift‡			>60	~40§	>60	>50
48 hr shift‡			>50	~45§	>50	>50

* Cells were incubated in depleted medium at the temperature and for the times indicated, after which complete medium was added at the same temperature.
† Complete medium added at the time of temperature shift.
‡ Cultures left in depleted medium and shifted from 40° to 33°.
§ The slope for the incorporation of thymidine under these conditions is very broad, as illustrated in Fig. 6.

check the validity of this treatment of the data, DNA synthesis was again followed in randomly growing cells, but after the addition of [^{3}H]thymidine at the time corresponding to 2.5 days in Fig. 1 or 1.5 days in Fig. 2. In each case (Fig. 3), the theoretical curve fit precisely the experimental points. We conclude that our theoretical treatment of the data is valid for randomly growing cultures, and can be used as an indication of the "randomness" of any culture.

The correspondence of the theoretical curve (labeled *R* in Fig. 3, upper left-hand corner) and the experimental points indicate that the wild-type transformed cells were randomly distributed throughout the cell cycle after 2 days in isoleucine-depleted medium. Similar results were obtained for *tsA239* transformed cells at 33°, although the experimental points do not lie precisely on the theoretical curve (Fig. 3, upper right-hand corner). However, if some of the cells were dead at the time of addition of complete medium (shedding had just started by 2.5 days), then the value of n determined from Fig. 1 would have been too high, and the experimental data fit a curve of lower n.

On the other hand, incorporation of thymidine into isoleucine-deprived, normal CHL cells growing at 33° or *A239* transformed cells growing at 40° occurs in a synchronized fashion. The data in both cases varied significantly from the theoretical curves, which assume random distribution at the time of addition of the radioactive label (Fig. 3). However, the synchrony was not perfect since significant incorporation was observed immediately after the addition of radioactive medium. It is thus impossible to determine precisely the onset of DNA synthesis. Approximate times of onset can be estimated from extrapolation of the steeply ascending portions of the experimental curves, but small deviations in the experimental points drastically affect such estimates.

Normal CHL and *A239* transformed cells were at least partially synchronized, as assayed by thymidine incorporation, not only when deprived of isoleucine, but also when deprived of glutamine or serum for 1 or 2 days at 40° (Fig. 4). Rough estimates of the interval from the addition of complete medium to the onset of DNA synthesis obtained by extrapolation of the data in Fig. 4 and and similar experiments are presented in Table 1. In many cases the length of this interval approached the generation time of the cells, thus exceeding the normal length of G_1. For example, cells transformed by *A239* with g = 18 hr at 40° did not initiate DNA synthesis in complete medium until 20 hr after 2 days of serum depletion.

In each case the onset of DNA synthesis appeared earliest in glutamine-deprived cultures, somewhat later in isoleucine-deprived cultures, and latest in cultures incubated in 0.5% serum. However, in no case was the difference in the onset as great as has been reported for baby hamster kidney cells (18). The greatest difference seen was between CHL cells depleted 2 days at 33° of glutamine (12 hr) and cells deprived of serum (20 hr). Although these results are consistent with the interpretation that serum deprivation results in an "early G_1 block," whereas isoleucine deprivation leads to a "mid-G_1 block" (18), we believe this interpretation to be incorrect. As pointed out by Pardee (20), if this interpretation were correct, then cells shifted from isoleucine-deprived medium to medium with little serum should traverse one *S* phase.

CHL cells shifted from one depleted medium to another did not enter *S* phase, corroborating the results of Pardee (20). Fig.

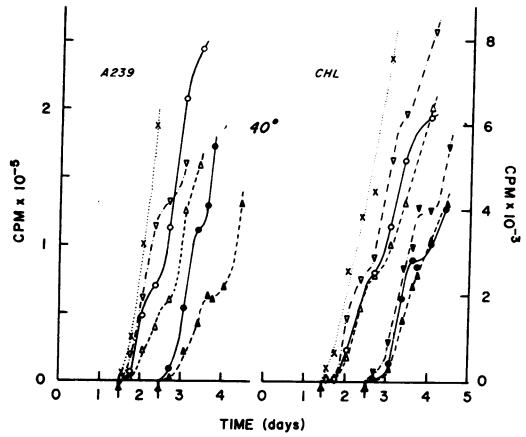

FIG. 4. Thymidine incorporation in normal CHL and *A239* transformed cells after amino acid or serum depletion at 40°. Cells were incubated in complete medium or depleted medium as in Fig. 2 for 24 or 48 hr. Complete radioactive medium was added at the times indicated by the arrows. Thymidine incorporation into cells that had not been depleted (×) or that had been depleted of glutamine (▽, ▼), isoleucine (○, ●), or serum (△, ▲) for 24 (open symbols) or 48 (closed symbols) hr was determined. The dotted lines were theoretically calculated for k = 0.5 cpm/cell for *A239* transformed cells and k = 0.35 cpm/cell for CHL cells using the values of g and n from the data in Fig. 2 and Fig. 6.

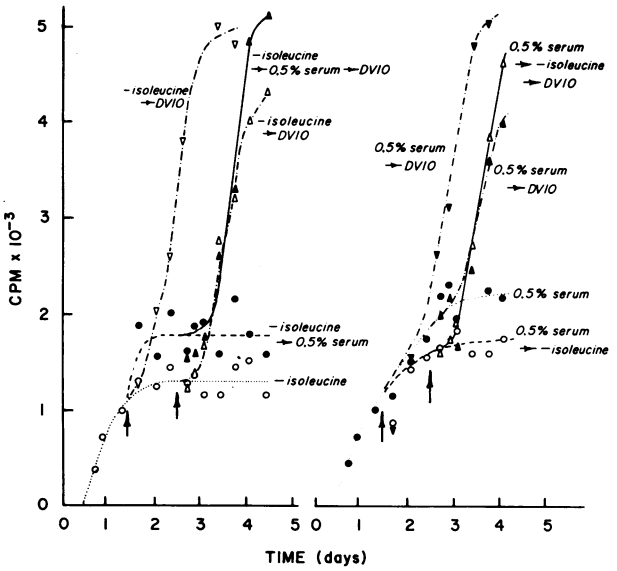

FIG. 5. Thymidine incorporation in CHL cells at 40° upon shifting from one depleted medium to another. Medium was replaced at 0.5 day with radioactive medium depleted of isoleucine (○) or serum (●). At the time indicated by the first arrow (approximately 1 day), the medium was changed to complete medium (▽, ▼), left in the same depleted medium, or shifted to the opposite depleted medium (●, ○). At the time indicated by the second arrow (approximately 2 days) the medium was again either left unchanged or replaced with complete medium (△, ▲). DV10, medium supplemented with 10% fetal bovine serum.

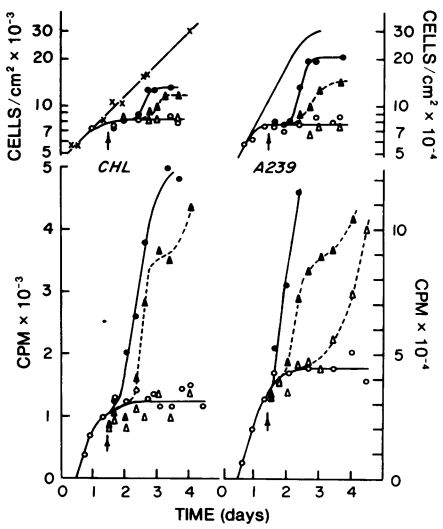

FIG. 6. Growth and thymidine incorporation of resting CHL and *A239* transformed cells upon temperature shift from 40° to 33°. Growth curves are as in Fig. 2. The experimental points for the randomly growing *A239* transformed cells are given in Fig. 2. After 24 hr of isoleucine depletion (○) in radioactive medium, the cells were either left in this same medium at 40° or shifted in this medium to 33° (△). Alternatively, the medium was replaced with complete medium and the incubation continued at 40° (●) or at 33° (▲).

5 illustrates the results when medium depleted of isoleucine (or serum) was added to randomly growing cells and then changed to medium depleted of serum (or isoleucine). From the value of k for these cells at 40° (Fig. 4) and the cell number, it can be calculated that a single division of the entire population should result in the incorporation of 2500 cpm. As can be seen, approximately 3500 cpm were incorporated in the first "step" after the addition of complete medium to cells deprived (*i*) of isoleucine (or serum) for 24 or 48 hr or (*ii*) of isoleucine (or serum) for 24 hr followed by serum (or isoleucine) for an additional 24 hr. However, shifting the cultures from isoleucine-deprived medium to serum-deprived medium resulted in the incorporation of only an additional 500 cpm. Similar results have been obtained at 40° with all combinations of shifting normal CHL cells or *A239* transformed cells to and from glutamine-deprived, isoleucine-deprived, and serum-deprived media. An apparent inconsistency exists between these results and those of Table 1 if it is assumed that resting cells are blocked in G_1. (Note: the term G_1 is used to refer to the progress towards S and not the state of all cells with an unduplicated genome.)

A further inconsistency in the notion that resting cells are arrested in the pathway from mitosis to S phase is provided by an analysis of the release of *A239* transformed cells from G_0 in temperature-shift experiments. As seen in Fig. 1, *A239* transformed cells grow at 33° with a nearly normal g in isoleucine-depleted medium up to cell densities approaching 2×10^5 cells per cm^2. Independent data (not presented) similarly demonstrate that thymidine incorporation proceeds in isoleucine-depleted medium at almost the same rate as in complete medium to this cell density. Hence, if G_0 were a point somewhere in G_1, then upon shifting a resting culture of *A239* transformed cells at 40° to 33°, one should find that thymidine incorporation was similar whether the medium remains depleted of isoleucine or if complete medium is added at the time of the shift down (provided the cell density is less than 2×10^5 cells per cm^2). Experimentally the onset of DNA synthesis upon shift down in depleted medium is greatly delayed (Fig. 6).

DISCUSSION

Cells transformed by the *tsA239* mutant of SV40 are temperature-sensitive for many characteristics generally associated with transformation (6, 11, 23). Our results confirm that, in addition, these cells behave like "normal" cells at the restrictive temperature in that they can enter a resting state, but are transformed at the permissive temperature in that they are unable to enter such a state.

A slightly modified view of how cells enter G_0, presented in Fig. 7, is consistent with the data presented and that of other workers referred to in the introduction of this paper. According to this model, a normal cell in complete or depleted medium proceeds through G_1 to the restriction point (20), which, we propose, immediately precedes the initiation of DNA synthesis. In suboptimal conditions the normal cell then proceeds out of the cell cycle to G_0. Some protein synthesis is presumably required for this transition so that cells deprived of amino acids may take longer to reach G_0 than cells deprived of serum factors. Whether addition of complete medium merely reverses this transition or whether the cell must return to early or mid-G_1 cannot be operationally distinguished at this time.

This model can resolve apparently contradictory data. As

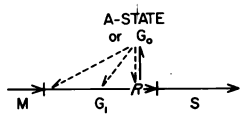

FIG. 7. The resting state, G_0 or the *A*-state (21, 22), is proposed to lie outside the normal cell cycle ($M \equiv$ mitosis, $G_1 \equiv$ pre DNA synthesis phase of growth, $S \equiv$ DNA synthesis). It is proposed that cells only enter G_0 after reaching a point late in G_1, the restriction point R (20). Reentry into the cell cycle is postulated to occur either by direct reversal of the R to G_0 transition or by a return to some point in G_1.

Burstin *et al.* have shown for baby hamster kidney cells (18) and is demonstrated here for CHL cells (Table 1), the interval from the time of addition of complete medium to the onset of DNA synthesis depends upon whether the cells are depleted for serum factors or amino acids. On the other hand, when cells are shifted from one type of depleted medium to another, they do not enter S phase (Fig. 5 and ref. 20), suggesting that they are all blocked at the same point (20). The conflict could be resolved by the proposed model if amino acid deprivation slowed or blocked the transition from R to G_0.

Another inconsistency in the notion of "mid" or "early" G_1 block that can be resolved by the model is that the interval from the restoration of complete medium to resting cells to the onset of DNA synthesis is often much greater than the length of G_1 (Table 1). This is explained by the model since the time interval measured by this type of experiment is that from G_0 to S, not from mid G_1 to S. Similar observations and conclusions have been reached by others (19, 24).

In addition, the model is consistent with the behavior of SV40 *tsA* mutant transformed cells placed in the resting state at the restrictive temperature and then shifted to the permissive temperature in depleted medium. In these experiments (Fig. 6) the onset of DNA synthesis is greatly delayed although the rate of growth of *A239* transformed cells in isoleucine-depleted medium at 33° is nearly the same as in complete medium (Fig. 1). (That DNA synthesis eventually resumes may indicate that a small fraction of the cells had not entered G_0 and overtook the culture at late times or that the resting cells may occasionally return to R.) It should be noted that these cells are T-antigen positive at the restrictive temperature (23) and that in permissive, *A239* infected cells, SV40 DNA synthesis resumes within 20 min after cultures are shifted to the permissive temperature (4). We therefore do not believe that the long lag can be explained as the length of time to resynthesize active T-antigen in depleted medium. These results are consistent with the model because the model suggests that the abnormality in SV40 transformed cells is not in their ability to remain in a resting state, so much as in their ability to enter such a state. No such distinction follows from the mid-G_1 block model.

Finally, the model is consistent with that of Burns and Tannock (21) and Smith and Martin (22) if normal cells in tissue culture enter the resting state with high probability.

None of the results presented in this paper defines the precise location of the restriction point within G_1. However, if the restriction point immediately precedes the S phase, then transformation by SV40 could be the result of the A gene product serving as an initiator of DNA synthesis to bypass the restriction point (11).

The model makes several predictions, including that some cell functions may exist that are only activated when cells enter G_0. In addition, it predicts that if cells are collected in mitosis and plated in depleted medium, then the interval of time from the reintroduction of complete medium to the onset of DNA synthesis should first decrease and then increase to a plateau as a function of the length of incubation in depleted medium

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