Reprinted from JOURNAL OF CELLULAR PHYSIOLOGY
Vol. 85, No. 1, February 1975

Isolation and Characterization of Revertant Cell Lines

VII. DNA SYNTHESIS AND MITOTIC RATE OF SERUM-SENSITIVE REVERTANTS IN NON-PERMISSIVE GROWTH CONDITIONS [1]

ARTHUR VOGEL AND ROBERT POLLACK
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

ABSTRACT The ability to synthesize DNA and enter mitosis was studied in Balb/c and Swiss 3T3 cells, SV40 and MSV-transformed 3T3 cells and revertants of these transformed cells in cultures of different serum concentrations and cell densities. Three ways were found by which cells were able to maintain a constant cell number in non-permissive growth conditions: cessation of DNA synthesis, synthesis of DNA coupled with failure to enter mitosis, and the slow traverse of the cell cycle coupled with cell shedding.

Growth control of the revertant of an MSV-transformed Balb/3T3 cell most closely resembled that of Balb or Swiss 3T3. This line did not grow in 1% serum and did not synthesize DNA in either non-permissive condition.

Serum-sensitive revertants of SV40-transformed 3T3 cells are also unable to grow in 1% serum and also do not grow beyond confluence in 10% serum, but these cells differ from 3T3 in the manner in which this growth arrest is accomplished. In 1% serum, revertants synthesize DNA but do not enter mitosis. At confluence in 10% serum, they slowly traverse the cell cycle, with dividing cells replacing cells that are shed into the medium.

When a cell population ceases to increase in number but does not die, it is said to be responding to growth control. In order for a population to increase in cell number, cells must replicate their DNA and then successfully complete mitosis. When a population is not increasing in number, the cells may be unable to synthesize DNA, they may synthesize DNA but be unable to enter mitosis, or division may be completed, with dividing cells replacing cells that detach from the monolayer and shed into the medium.

The 3T3 mouse fibroblast lines isolated from either Balb/c or Swiss mice exhibit a high degree of growth control (table 1). 3T3 cells do not grow in low amounts of serum (Todaro et al., '67; Holley and Kiernan, '68; Dulbecco, '70), are unable to grow beyond a confluent monolayer in 10% calf serum (Todaro and Green, '63; Black, '66), and do not form colonies when suspended in medium containing methylcellulose (Black, '66). Transformation by SV40 (Risser and Pollack, '74) or murine sarcoma virus renders 3T3 cells relatively insensitive to the regulation of proliferation imposed by these restrictive conditions (table 1). The transformed cells grow in low serum concentration, grow beyond confluent cell densities in 10% serum, and grow into colonies when suspended in methocel (Todaro et al., '64; Black, '66; Holley and Kiernan, '68; Dulbecco, '70; Jainchill and Todaro, '70; Aaronson and Rowe, '70; Smith et al., '71; Risser and Pollack, '74; Ozanne and Vogel, '74).

Serum-sensitive and density-sensitive revertants have been isolated which have reacquired growth control (table 1). The density-sensitive revertant F1SV101 responds to confluence by reductions in the rate of DNA synthesis and in the fraction of cells entering mitosis (Pollack and Vogel, '73) but is not sensitive to low serum (Vogel and Pollack, '73). In this paper we examine the ability of 3T3 cells, SV40 and MSV-transformed 3T3 cells and serum-sensitive revertants of the transformed 3T3 cells to synthesize DNA and enter mitosis when subjected to two restrictive

Received June 17, '74. Accepted Aug. 13, '74.

[1] Paper VI of this series is A. Vogel and R. Pollack, "Isolation and Characterization of Revertant Cell Lines. VI. Susceptibility of Revertants to Retransformation by SV40 and Murine Sarcoma Virus," in press, in the *Journal of Virology*.

TABLE 1

Growth properties of cell lines derived from 3T3 cells

Cell lines	Transforming virus	Saturation density Cells/cm² × 10⁴		Doubling time (hrs)	
		1%	10%	1%	10%
Normal					
Swiss 3T3	—	1–2	5–8	90	21
Balb/3T3	—	N.T.	8–10	77	22
Transformed					
SV101	SV40	10	>45	30	16
KA31	Ki-MSV	N.T.	>50	38	19
Serum revertants (from SV101)					
LsSV2		—	10–15	>120	22
AγSV4		—	10–15	>120	22
Density revertant (from SV101)					
F1SV		2	7–9	36	22
Methocel revertant (from KA31)					
M22		—	7	98	29

culture conditions which do not support their net cell increase: confluence and low serum.

3T3 and M22, a revertant of an MSV-transformed Balb/3T3 non-producer cell (Ozanne and Vogel, '74) behave alike, failing to synthesize DNA in sparse culture in 1% serum or at confluence in 10% calf serum. Hence these cells are arrested early in the cell cycle by both restrictive conditions.

Serum-sensitive revertants of SV3T3 (Vogel and Pollack, '73) respond differently to the two non-permissive conditions. In 1% serum, they synthesize DNA but do not enter mitosis. At confluence in 10% serum, the serum-sensitive revertants slowly traverse the cell cycle and a constant number of cells per dish is maintained by dividing cells replacing cells which have died and shed.

MATERIALS AND METHODS

Stock cultures

Stock cultures were routinely grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.) and 50 μg/ml Gentamycin (Schering) in 28 cm² tissue culture dishes (Falcon). The cells were kept at 37°C in a water saturated 10% CO_2-90% air atmosphere.

Revertant cell lines

Variant sublines have been isolated from SV40 or MSV-transformed 3T3 cells which have reverted to a 3T3-like inability to grow in certain restrictive growth conditions (Pollack et al., '68; Ozanne and Sambrook, '71; Culp et al., '71; Culp and Black, '72; Nomura et al., '72, '73; Ozanne, '73; Vogel and Pollack, '73; Vogel et al., '73; Greenberger and Aaronson, '74; Ozanne and Vogel, '74). The growth properties of these revertants in 1% and 10% calf serum are summarized in table 1.

The density-sensitive revertant F1SV101 was isolated from SV101, a clone of SV40-transformed Swiss/3T3 cells (Pollack et al., '68). It was selected for the inability to grow to high cell density in 10% serum by plating cells at high cell density and killing dividing cells with Fudr.

The serum-sensitive revertants, LsSV2 and AγSV4, were also derived from SV101. LsSV2 was selected for its inability to grow in 1% calf serum by plating the cells at sparse densities in 1% serum and killing the dividing cells with Budr (Vogel and Pollack, '73). AγSV4 was isolated in a similar fashion, except the non-permissive serum was 10% gamma-globulin free depleted newborn calf serum (Smith et al., '71). LsSV2 or AγSV4 do not grow in 1% calf serum and do not grow beyond confluent cell densities in 10% serum (Vogel and Pollack, '73).

M22 is a revertant of KA31, a Kirsten-MSV transformed Balb/3T3 non-producer cell (Aaronson and Rowe, '70). M22 was selected to be unable to grow in Methocel medium, by plating KA31 in medium containing methylcellulose, and adding Fudr

to kill dividing cells (Ozanne and Vogel, '74).

Cell growth

Cell number was determined by trypsinizing a plate of cells and counting the cells in a Coulter Counter. The medium was changed every 3 days in all growth determinations.

Incorporation of ^{3}H-Tdr into DNA and autoradiography of ^{3}H-Tdr

Cells were plated in medium containing 1% or 10% calf serum and 10^{-5} M cold Tdr in 35 mm dishes containing 12 mm circular coverslips. Two days after the final medium change, ^{3}H-Tdr (5–10 μCi/ml, >15 Ci/mM, NEN) was added. At various times after the addition of label, coverslips were removed, washed by successive dips in PBS, fixed in ethanol-acetic acid (2:1), air dried and mounted cell side up on microscope slides. The slides were dipped in melted (40°C) Kodak NTB-2 emulsion, air dried for two hours, and stored at 4°C for two days. The autoradiographs were developed in Dektol, and allowed to dry overnight. They were then stained in Hematoxylin (Harris), blued in $LiCl_2$, washed in water, and mounted in elvanol. The fraction of labelled nuclei was determined at 400 × on a Zeiss phase microscope, with 300–500 nuclei counted for each determination. The data is plotted as fraction of cells labelled versus time in ^{3}H-Tdr.

For labelling experiments done in sparse culture in 1% calf serum, the cells were plated at 0.1–0.2 × 10^4 cells per cm^2 and label was added 48 hours later. Labelling experiments done at confluence were started by seeding the cells at 1–2 × 10^4 cells/cm^2 and allowing the cells to grow to confluence. When the cells reached confluence, the medium was changed and label added 48 hours after the medium change.

Fraction of cells in mitosis

Cells were incubated with 0.01 μg/ml Velban (Gibco) for 2–4 hours. The medium was removed, the cells trypsinized, and the medium and trypsinized cells were pooled and centrifuged. The cells were swelled in 0.38% KCl, fixed in methanol-acetic acid (3:1) overnight and spread on clean slides. The slides were stained with Giemsa, dehydrated in successive ethanol baths and xylene, and mounted in Permount. The percent mitotic cells was determined at 400 × on a Zeiss phase microscope, with 100–1000 cells counted.

For mitotic rates in 1% serum, the cells were plated at 0.2–0.4 × 10^4 cells/cm^2 and incubated for 48 hours. Velban (0.01 μg/ml) was then added and the mitotic cells collected for 2–4 hours and processed as described above.

For mitotic rates done at confluence in 1% or 10% serum, the cells were plated at 1–2 × 10^4 cells/cm^2 and allowed to grow to confluence. The medium was changed and 48 hours later, Velban was added for two hours to collect mitotic cells.

For shift experiments in which the cells were shifted from 1% to 10% calf serum, the cells were plated in 1% calf serum at densities of 0.2–0.4 × 10^4 cells/cm^2. Two days later, fresh calf serum was added to a final concentration of 10% and mitotic cells were collected with Velban (0.01 μg/ml) 0–3, 4–7, 7–10, 10–14, and 14–18 hours after the addition of fresh serum. The mitotic cells were processed as described above.

Labelled mitoses

Cells were plated in 1% calf serum containing 10^{-5} M Tdr and 48 hours later the cells were pulse labelled for one hour with 5 μCi/ml of ^{3}H-Tdr (>15 Ci/mM, NEN). After the pulse, the medium was removed and fresh 1% or 10% calf serum medium containing 10^{-5} M cold Tdr was added to the cells. Mitotic cells were collected 0–4, 4–7, and 7–10 hours after the one hour pulse. The mitotic cells were processed as described above, dipped in melted Kodak NTB-2 emulsion, incubated for 9 days at 4°C and developed in Dektol. The slides were stained with Hematoxylin and the fraction of labelled mitosis determined. Thirty to sixty mitoses were examined.

PPLO contamination

The serum revertants LsSV2 and AγSV4 were found to be contaminated with PPLO by the criterion of extranuclear grains after ^{3}H-Tdr autoradiography. The addition of Kanamycin (100 μg/ml) (Gibco) and Lincomycin (500 μg/ml) (Gibco) suppressed the appearance of extranuclear

grains (Nardone et al., '65). The addition of these two antibiotics to cultures of PPLO free cells had no effect on the incorporation of ^{3}H-Tdr (Vogel, unpublished results). Therefore, all labelling experiments were done in the presence of Kanamycin and Lincomycin. These drugs did not cure the cultures of PPLO, but suppressed the incorporation of thymidine into the PPLO (Vogel, unpublished results; Nardone et al., '65).

RESULTS

Response to serum restriction

Untransformed cells depend on serum for the initiation of DNA synthesis. In 1% serum, both Swiss and Balb/3T3 cells do not synthesize DNA (fig. 1a,b). The transformed cells, SV101 and KA31, synthesize DNA and grow in 1% serum (fig. 1c,d). In fact, SV101 cells synthesize DNA in 0.1% calf serum even though they do not increase in cell number (fig. 1c). As described previously, the density-sensitive revertant F1SV synthesizes DNA and grows in 1% serum (Vogel and Pollack, '73) (fig. 1e).

The three revertants which do not grow in 1% serum exhibit two distinct types of growth behavior. M22, the revertant of KA31, behaves like 3T3 since it does not synthesize DNA (fig. 1f). The serum-sensitive revertants LsSV2 and AγSV4 synthesize DNA in 1% calf serum. Approximately 50% of the cells are labelled after 12 hours in ^{3}H-Tdr and 80–90% are labelled after 48 hours (fig. 1g,h).

Serum shift experiments were performed to determine when 3T3 or M22 cells begin to synthesize DNA. If 3T3 or M22 cells are shifted from 1% to 10% serum, the fraction of cells synthesizing DNA in 10% does not increase beyond the fraction synthesizing DNA in 1% serum until 20–25 hours after shift. The cells double only by 40 hours after shift (fig. 2a,b,c). Thus, in 1% serum, 3T3 and M22 cells are blocked in the cell cycle prior to S.

Since the serum-sensitive revertants LsSV2 and AγSV4 make DNA but do not increase their number on a dish in 1% serum, we compared these cells to SV101 and 3T3 for the ability to enter mitosis in various serum concentrations. 3T3 cells did not enter mitosis in 1% serum, while SV101 and F1SV showed mitotic frequencies of approximately 2% per hour (table 2). In 0.1% serum, SV101 cells are unable to grow (fig. 1c), and they enter mitosis at a very low rate (table 2).

The serum revertant LsSV2 has a low mitotic frequency in 1% calf serum, comparable to the mitotic frequency of SV101 in 0.1% serum (table 2). The other serum revertant, AγSV4, also displays a decrease in mitotic rate, but the drop is not as great as in LsSV2.

To further characterize this inability to enter mitosis in low serum, we shifted the cells from 1% to 10% serum and observed when mitosis occurred. Since the cells are synthesizing DNA, mitosis should occur soon after the shift. Four to seven hours after serum addition, SV101 cells show a two-fold increase in mitotic frequency (table 3) and seven to ten hours after shift, all the cell lines show an increase in mitotic frequency (table 3). Thus, the addition of fresh serum allows these cells to enter mitosis. However, the addition of serum did not result in a large fraction of the cells synchronously entering mitosis, but rather restored the mitotic frequency to the rate seen in cultures grown in 10% serum. This increase in mitotic frequency occurs well before 3T3 and M22 cells shifted from 1% to 10% serum have started to synthesize DNA (fig. 2).

Mitoses were labelled to determine if the serum-sensitive cells were blocked in a specific part of the cell cycle. Any cell in G_2 at the time of addition of label should not incorporate any ^{3}H-Tdr into DNA and should show an unlabelled mitosis. Alternatively, any cell in S at the time of addition of label should incorporate the ^{3}H-Tdr into DNA and give a labelled mitosis. Thus, if the serum revertants are unable to complete S in 1% serum all the mitoses collected after addition of 10% serum should be labelled. If the cells are able to leave S, but are blocked in G_2, the mitoses should be unlabelled. Only 50% of the mitoses

Fig. 1 Cell growth and fraction of cells synthesizing DNA at sparse cell density. ^{3}H-Tdr was added 48 hours after the cells were plated (24 hour point). The cells were incubated in label for 48 hours. Coverslips were processed for autoradiography at varying times during this 48 hour period. (△), 0.1% calf serum; (○), 1% calf serum; (●), 10% calf serum. (a), Swiss/3T3; (b), Balb/3T3; (c), SV101; (d), KA31; (e), F1SV; (f), M22; (g), LsSV2; (h), AγSV4.

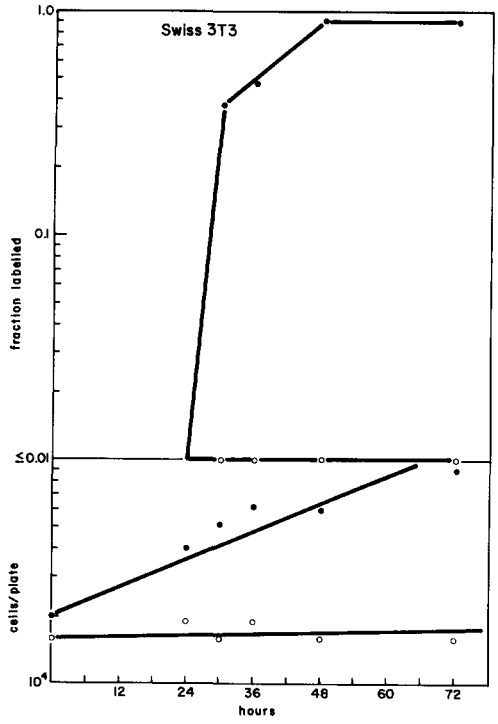

Figure 1a

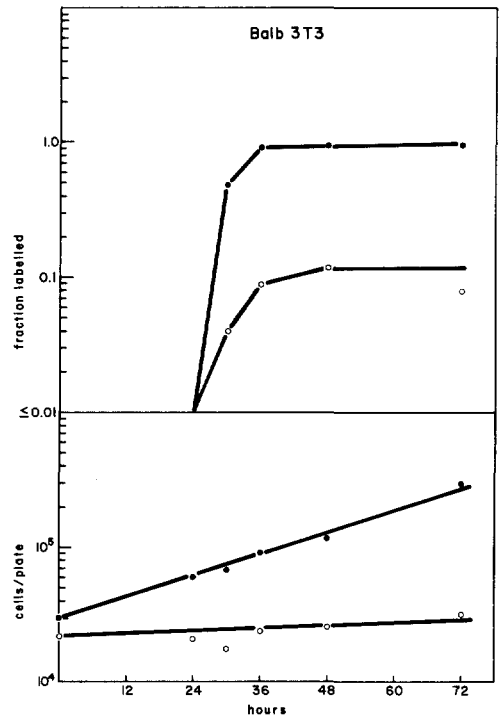

Figure 1b

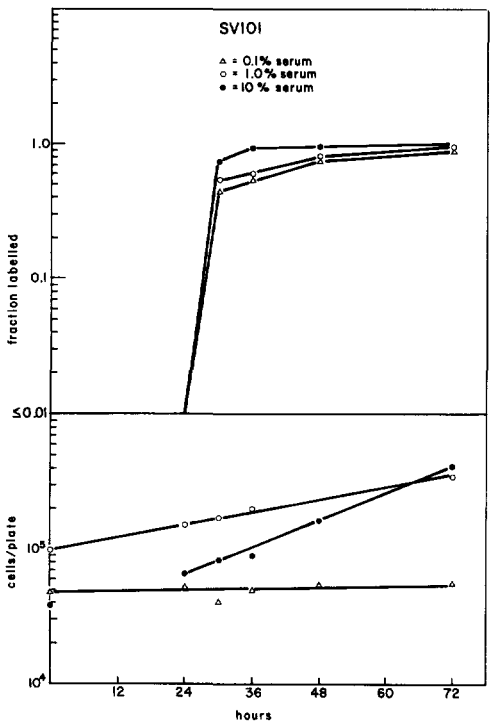

Figure 1c

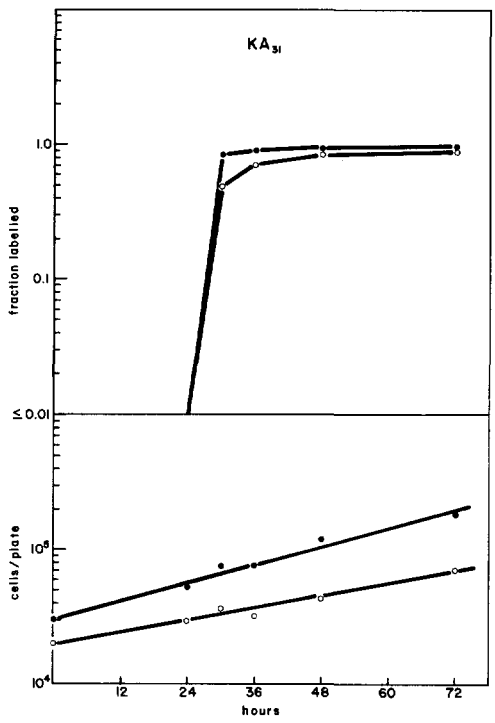

Figure 1d

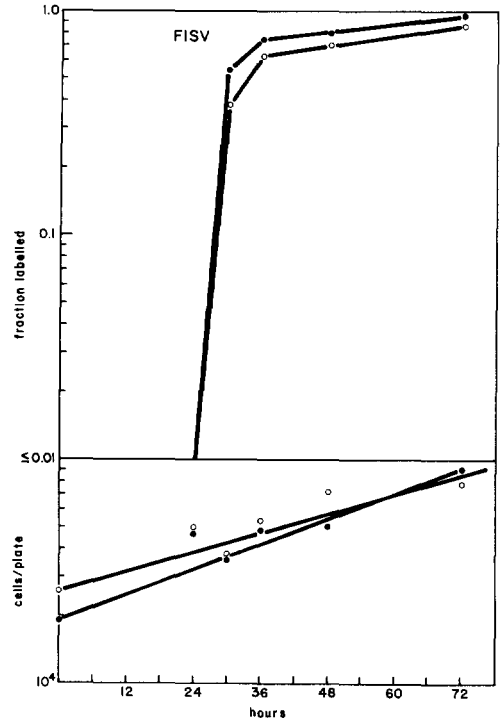

Figure 1e

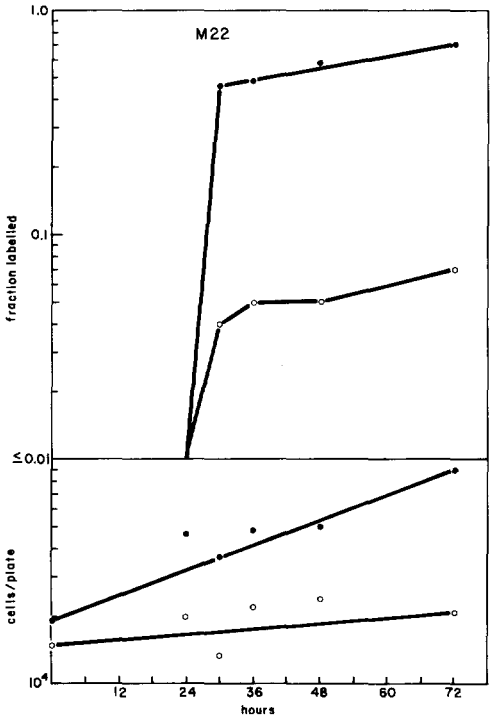

Figure 1f

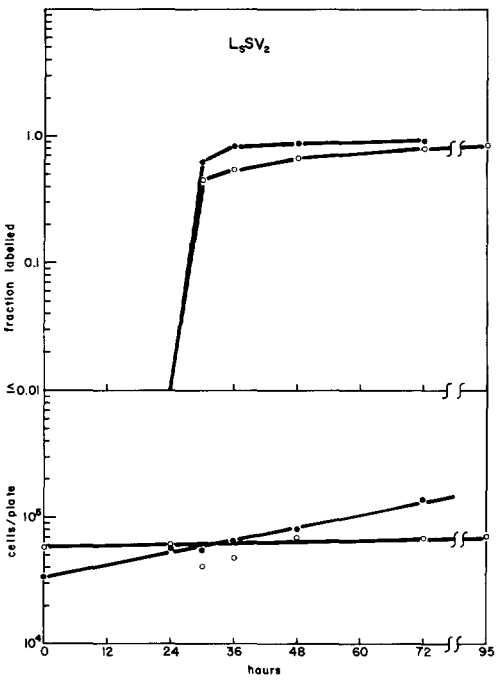

Figure 1g

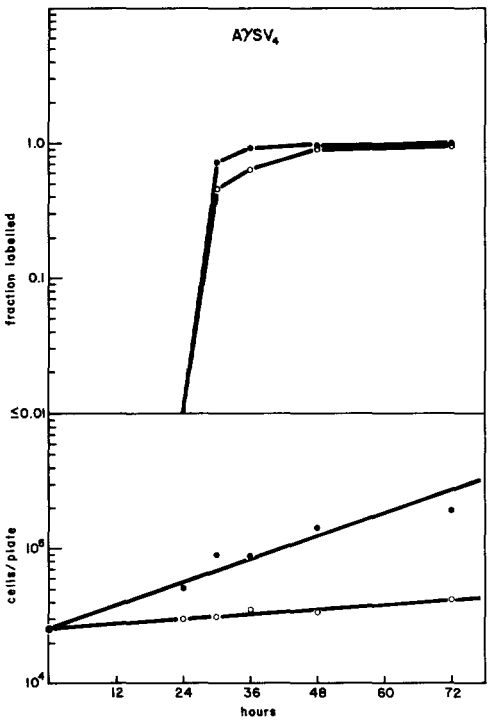

Figure 1h

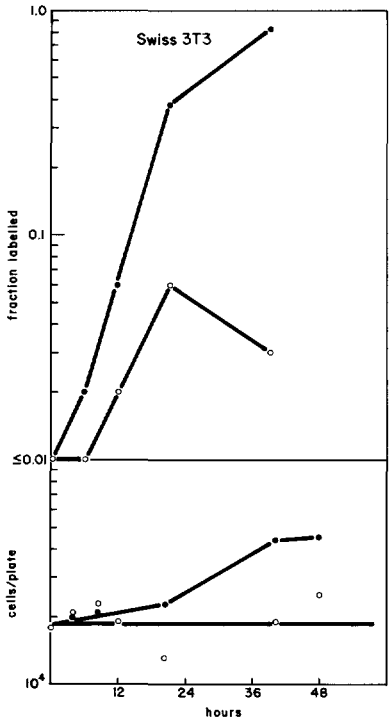

Figure 2a

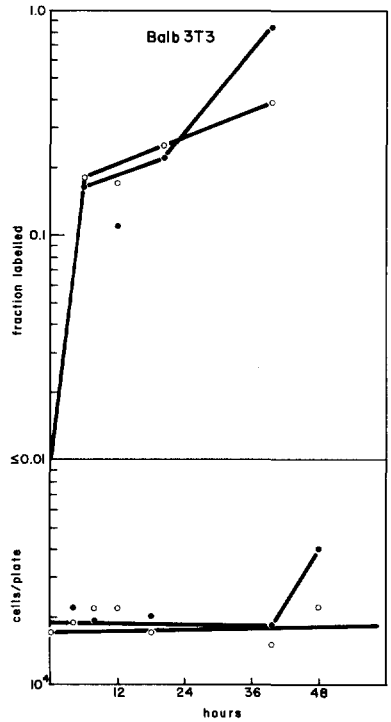

Figure 2b

were labelled 7 to 10 hours after the addition of 10% serum (table 4), implying that half the cells were in S and half the cells in G_2 at the time of addition of ^{3}H-Tdr. This result is consistent with the mitotic rate seen in cultures shifted from 1% to 10% serum. In 1% serum, the cells are in different parts of the cell cycle except M and shift to 10% serum allows the cells to enter mitosis at a rate characterisitc of cells growing logarithmically in 10% serum.

Response to density restriction

All of the revertants and 3T3 cells are unable to grow beyond a confluent monolayer in 10% calf serum, while the transformed cells continue to grow at high cell density (fig. 3a,b,c). Both Balb and Swiss 3T3 shut off DNA synthesis at confluence

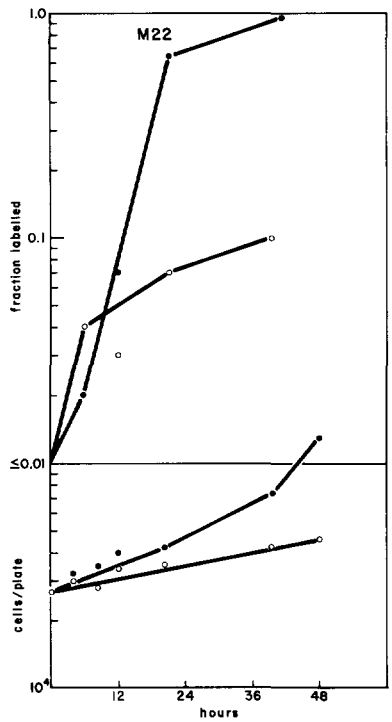

Figure 2c

Fig. 2 Cell growth and fraction of cells synthesizing DNA after shift from 1% to 10% calf serum. Fresh serum, at a final concentration of 10%, and ^{3}H-Tdr were added at 0 hours. The cells were left in label for 40 hours, with coverslips processed for autoradiography at varying time points. (O), 1% calf serum; (●), 1%→10% calf serum. (a), Swiss/3T3; (b), Balb/3T3; (c), M22.

TABLE 2

Mitotic frequency in varying serum concentrations

Cell line	Mitotic frequency [1] (Percent mitosis/hr) 0.1%	1%	10%	Ratio 10%/1%
Normal				
Swiss/3T3	N.T.[2]	0.1	3.0	30
Transformed				
SV101	0.6	2.0	3.5	1.75
Density-sensitive revertant				
F1SV	N.T.	1.8	3.0	1.67
Serum-sensitive revertant				
LsSV2	N.T.	0.5	2.9	5.8
AγSV4	N.T.	1.2	2.8	2.3

[1] Mitotic rates were determined two days after the cells were plated sparsely in 0.1%, 1% or 10% serum.
[2] N.T. = Not Tested.

(Nilhausen and Green, '65; Dulbecco, '70; Scher and Nelson-Rees, '71). At confluence, only 10% of the cells are labelled with ^{3}H-Tdr after 48 hours (fig. 3a). M22, the revertant of KA31 cells, also shuts off DNA synthesis (fig. 3a).

Cells of both the serum-sensitive revertants LsSV2 and AγSV4 and the density-sensitive revertant F1SV continue to incorporate thymidine at confluence (fig. 3c), as assayed by radioautography. It should be noted that although the majority of F1SV cells synthesize DNA at confluence, they do so at a rate which is 30-fold less than their rate of DNA synthesis in sparse culture (Pollack and Vogel, '73). Whether the serum-sensitive revertants also respond to confluence by diminishing their rates of DNA synthesis is unknown. However, it is clear from these data that none of our revertants derived from SV40-transformed cells are able to re-establish a density-sensitive block preceding S in the cell cycle.

These two types of revertants differ in their ability to enter mitosis. The density revertant F1SV has a lower mitotic frequency at confluence than either serum revertant (table 5). Both serum revertants LsSV2 and AγSV4 display the mitotic frequency at confluence (1–2% per hour) corresponding to a doubling time of 50–100 hours (table 5). While this frequency is 2–3 fold lower than the mitotic frequency of sparse, growing cultures, it is clear that the serum revertants proceed through the cell cycle at a slow rate even at confluence. We therefore hypothesize that cul-

TABLE 3

Mitotic frequency after shift from 1% to 10% calf serum

Time (hrs)	SV101 1%	SV101 10%	SV101 1→10%	F1SV 1%	F1SV 10%	F1SV 1→10%	LsSV2 1%	LsSV2 10%	LsSV2 1→10%	AγSV4 1%	AγSV4 10%	AγSV4 1→10%
						% *Mitosis/hr* [1]						
0–3	1.8	2.7	1.7	1.3	2.5	1.3	0.8	2.3	0.2	1.5	2.4	0.5
4–7	—	—	3.9	—	—	1.1	—	—	0.6	—	—	1.0
7–10	—	—	4.9	—	—	2.5	—	—	2.6	—	—	3.4
10–14	1.6	—	2.2	0.9	—	2.1	0.3	—	2.7	1.0	—	3.8
14–18	—	—	2.8	—	—	1.9	—	—	2.7	—	—	3.5

[1] Cells were plated sparsely in 1% or 10% calf serum. Two days later, fresh calf serum was added to a final concentration of 10% serum. Velban (0.01 μg/ml) was added and mitotic cells collected at varying times after serum addition.

TABLE 4

Fraction of labelled mitoses in mouse cell lines grown in different concentrations of serum

Time (hrs)	SV101 1%	SV101 1→10%	F1SV 1%	F1SV 1→10%	LsSV2 1%	LsSV2 1→10%	AγSV4 1%	AγSV4 1→0%
			Fraction labelled mitosis [1]					
0–4	0.40	0.20	0.33	0.36	<0.06	0.08	0.06	0.07
4–7	0.90	0.69	0.86	0.70	0.36	0.31	0.50	0.20
7–10	1.0	1.0	1.0	1.0	0.64	0.55	0.77	0.48

[1] The cells were pulse labelled in 1% calf serum for 1 hour, the label removed, and fresh medium containing 1% or 10% serum added. Mitotic cells were collected and autoradiographed as described in MATERIALS AND METHODS.

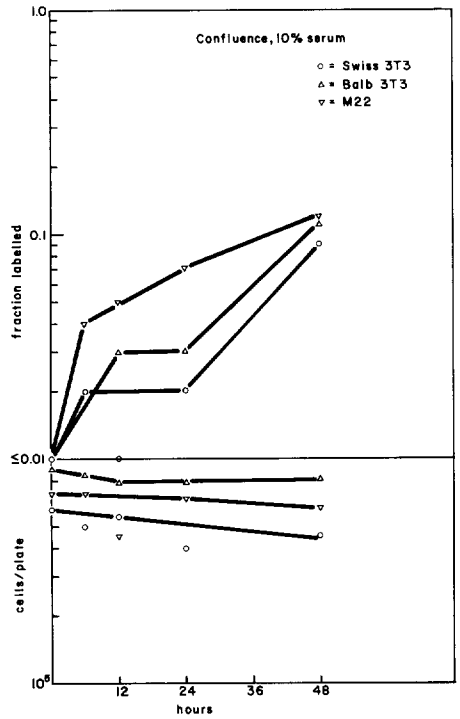

Figure 3a

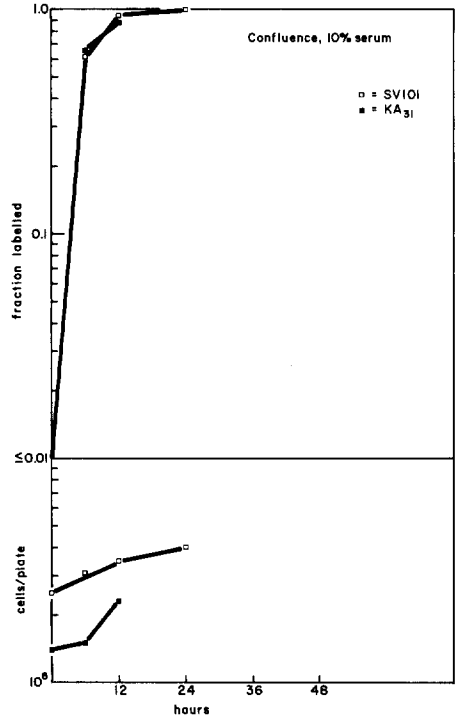

Figure 3b

tures of serum-sensitive revertants maintain their constant low saturation density by shedding cells into the medium at a rate equivalent to the reduced growth rate at confluence. This hypothesis is supported by the facts that many floating cells are observed in confluent cultures of LsSV2 and AγSV4, and that the number of floating cells increases as the cells sit at confluence.

A striking similarity exists in the way by which the serum revertants maintain a low saturation density in 10% serum and the way by which SV101 maintains a low saturation density in 1% serum. Although SV101 cells grow in 1% calf serum, they cease to increase in number at confluent cell densities (fig. 4). At confluence, both types of cells slowly traverse the cell cycle. The SV101 cells in the

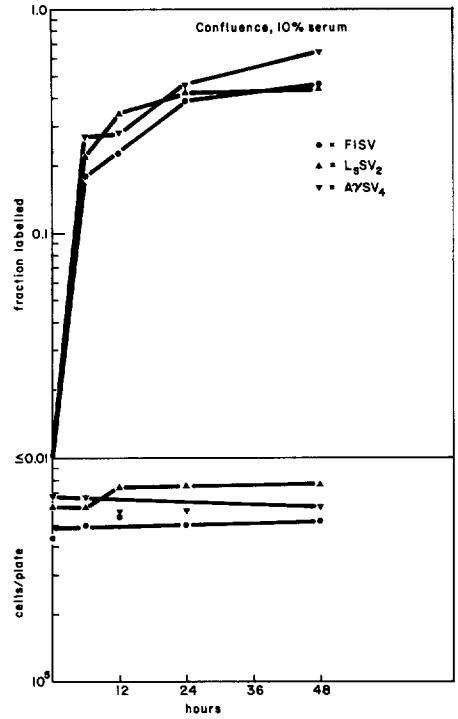

Figure 3c

Fig 3 Cell growth and fraction of cells synthesizing DNA at confluence in 10% serum. ^{3}H-Tdr was added at 0 hours and the cells were incubated in label for 48 hours, with coverslips processed for autoradiography at varying time points. (○), Swiss/3T3; (△, Balb/3T3; (▽), M22; (□), SV101; (■), KA31; (●), F1SV; (▲), LsSV2; (▼), AγSV4.

monolayer synthesize DNA (fig. 5) and enter mitosis at a frequency of 1% per hour, a frequency comparable to that of the serum revertants in 10% serum at confluence.

DISCUSSION

Cell populations selected for increased growth control maintain constant numbers in different ways. Some cell lines are arrested early in the cell cycle prior to DNA synthesis. 3T3 and M22 cells are examples of this form of growth control. Other lines make DNA but are unable to complete the cell cycle and enter mitosis. The serum revertants LsSV2 and AγSV4 in 1% serum, SV101 in 0.1% serum, and F1SV at confluence in 10% serum are examples of this type of growth regulation. A third type of growth regulation is manifested by LsSV2 and AγSV4 at confluence in 10% serum, and by SV101 at confluence in 1% serum. Here, the cells slowly traverse the cell cycle and constant cell number is probably maintained by cell division compensating for cells shed into the medium.

TABLE 5

Mitotic frequency of mouse cell lines at confluent saturation density in 10% calf serum

Cell line	Cell density Cells/cm² × 10⁴	Percent mitosis/hr [1]
Parent cell lines		
3T3	7.0	0.02
SV101	10.0	7.6
Serum revertants		
LsSV2	10.0	2.0
AγSV4	10.0	0.9
Density revertant		
F1SV	8.7	0.3

[1] Cells were allowed to reach confluence, the medium was changed, and mitotic rate determined 48 hours after the medium change.

DNA synthesis

3T3 cells display an absolute dependence on serum for the initiation of cellular DNA synthesis (Dulbecco, '70). In contrast to 3T3, the transformed cells and all revertants except M22 synthesize DNA in low concentrations of serum, even serum

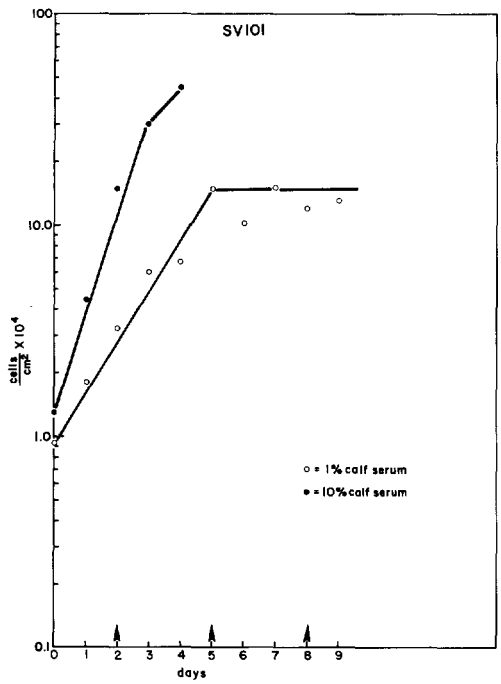

Fig. 4 Growth of SV101 in 1% and 10% calf serum. Medium was changed every three days (arrows). (○), 1% calf serum; (●), 10% calf serum.

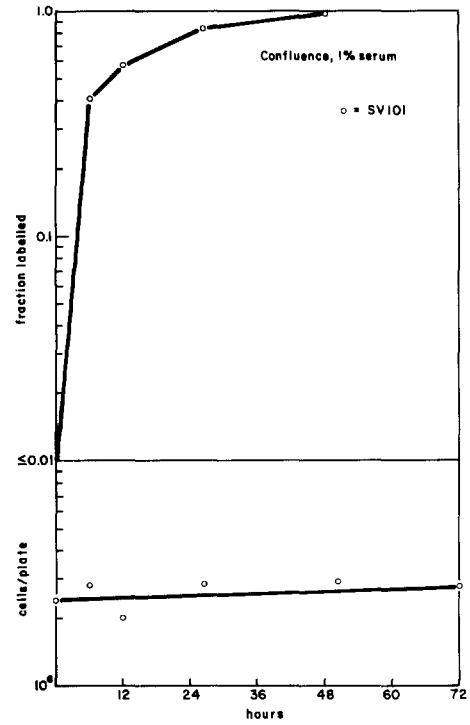

Fig. 5 Cell growth and fraction of cells synthesizing DNA at confluence in 1% calf serum. (○), SV101.

concentrations which do not support cell proliferation. This suggests that a major effect of transformation is to render 3T3 cells independent of serum for the initiation of cellular DNA synthesis.

Revertants of MSV-transformed 3T3 cells display different modes of growth control than revertants isolated from SV40-transformed 3T3 cells (Ozanne and Vogel, '74). M22 behaves identically to 3T3 cells in response to serum deprivation and confluence, demonstrating that a virus-transformed cell can revert to a form of growth control identical to that of the parent 3T3 cell. The revertants of SV40-transformed 3T3 cells display forms of growth arrest different from 3T3. It is interesting that a variant subline of SV40 transformed 3T3 cells which has reverted to a 3T3-like inability to synthesize DNA in non-permissive conditions has not yet been isolated. Perhaps the presence of the SV40 genome, or one of its gene products (such as T-antigen) in all of the revertants isolated so far, in some way prevents the re-establishment of growth control through the mechanism of a G_1-block.

Mitosis

While transformed cells do not require serum to initiate cellular DNA synthesis, they do need serum to enter mitosis and complete the cell cycle (Dulbecco, '70; Shodell, '72). Serum-sensitive revertants are likely to need more of this factor than does SV101. Both SV101 and the serum-sensitive revertants respond to serum deprivation similarly, except that the cessation of proliferation occurs at higher serum concentration in the revertants than in SV101. In 1% serum, the serum-sensitive cells appear to be stopped throughout both the S and G_2 phases of the cell cycle. Thus, addition of fresh 10% serum does not lead to a synchronized burst of cells undergoing mitosis, but rather results in the restoration of the mitotic frequency to the level seen in cells growing in 10% serum. This increase in mitotic frequency occurs seven hours after the serum shift.

PPLO infection cannot be responsible for the inability of serum-sensitive lines to grow in low concentrations of serum, since PPLO contaminated cultures of SV101 and F1SV grow as well in 1% calf serum as do PPLO free cultures of these cells and since cultures of PPLO contaminated 3T3 cells do not show any alteration in growth properties when compared with uncontaminated 3T3 cells (Vogel, unpublished results).

The final point concerns the role of cyclic AMP in the regulation of growth in these various cell lines. We have previously shown that when cells stop growing because of an insufficiency of serum, intracellular cyclic AMP levels markedly increase (Oey et al., '74). Both 3T3 and the serum-sensitive revertants respond in this way. However, even though 3T3 and the serum revertants behave similarly with respect to cyclic AMP levels and growth in 1% serum, they are different with respect to how they cease cell division. This difference raises questions about the role of the cyclic AMP in growth arrest. If the rise in cyclic AMP actually causes the cessation of cell proliferation, then cAMP must affect different cells in different parts of the cell cycle, since in 3T3 the high cyclic AMP level leads to a cessation of cellular DNA synthesis, while in the serum revertants, it decreases the mitotic frequency. Alternatively, the increase in cyclic AMP level may not play a primary role in the cessation of cell division, but may be a cellular event which occurs after the cells have received another signal which determines the cessation of cell division. In this model, cyclic AMP levels increase only after some other primary signal has determined that the cells stop growing.

ACKNOWLEDGMENTS

We thank Rex Risser and Robert Goldman for their helpful discussions, and Sue Conlon and Carole Thomason for excellent technical assistance.

A. V. is supported by NIH training grant 5 T05 GM01668 from the National Institute of General Medical Science. R. P. is supported by Public Health Service research grant CA-08888 from the National Cancer Institute. This work was supported by NIH cancer center grant 1 P01 CA13106.

LITERATURE CITED

Aaronson, S., and W. Rowe 1970 Non-producer clones of murine sarcoma virus transformed Balb/3T3 cells. Virology, 42: 9–19.

Black, P. H. 1966 Transformation of mouse cell line 3T3 by SV40: Dose response relationship and correlation with SV40 tumor antigen production. Virology, *28*: 760–763.

Culp, L., and P. Black 1972 Contact-inhibited revertant cell lines isolated from simian virus 40-transformed cells. III. Concanavalin A-selected revertant cells. J. Virol., *9*: 611–620.

Culp, L., W. Grimes and P. Black 1971 Contact-inhibited revertant cell lines. I. Biologic, virologic and chemical properties. J. Cell Biology, *50*: 682–690.

Dulbecco, R. 1970 Topoinhibition and serum requirement of transformed and untransformed cells. Nature, *227*: 802–806.

Greenberger, J., and S. Aaronson 1974 Morphologic revertants of murine sarcoma virus transformed non-producer Balb/3T3: Selective techniques for isolation and biologic properties in vitro and in vivo. Virology, *57*: 339–346.

Holley, R., and J. Kiernan 1968 "Contact inhibition" of cell division in 3T3 cells. Proc. Nat. Acad. Sci., *60*: 300–304.

Jainchill, J., and G. Todaro 1970 Stimulation of cell growth in vitro by serum with and without growth factor; relation to contact inhibition and viral transformation. Exp. Cell Res., *59*: 137–146.

Nardone, R., J. Todd, P. Gonzalez and E. Gaffney 1965 Nucleoside incorporation into strain L cells: inhibition by pleuropneumonia-like organisms. Science, *149*: 1100–1101.

Nilhausen, K., and H. Green 1965 Reversible arrest of growth in G_1 of an established fibroblast line (3T3). Exp. Cell Res., *40*: 166–168.

Nomura, S., P. J. Fischinger, C. Mattern, B. Gerwin and K. Dunn 1973 Revertants of mouse cells transformed by murine sarcoma virus. II. Flat revertants induced by fluorodeoxyuridine and colcemid. Virology, *56*: 152–163.

Nomura, S., P. Fischinger, C. Mattern, P. Peebles, R. Bassin and G. Friedman 1972 Revertants of mouse cells transformed by murine sarcoma virus. I. Characterization of flat and transformed sublines without a rescuable murine sarcoma virus. Virology, *50*: 51–64.

Oey, J., A. Vogel and R. Pollack 1974 Intracellular cyclic AMP concentration responds specifically to regulation by serum. Proc. Nat. Acad. Sci., *71*: 694–698.

Ozanne, B. 1973 Variants of simian virus 40-transformed 3T3 cells that are resistant to concanavalin A. J. Virol., *12*: 79–89.

Ozanne, B., and J. Sambrook 1971 Isolation of lines of cells resistant to agglutination by concanavalin A from 3T3 cells transformed by SV40. Lepetit Colloq. Biol. Med., *2*: 248–253.

Ozanne, B., and A. Vogel 1974 Selection of revertants of Kirsten sarcoma virus transformed non-producer Balb/3T3 cells. J. Virol., *14*: 239–248.

Pollack, R., H. Green and G. Todaro 1968 Growth control in cultured cells: Selection of sublines with increased sensitivity to contact inhibition and decreased tumor-producing capacity. Proc. Nat. Acad. Sci., *60*: 126–133.

Pollack, R., and A. Vogel 1973 Isolation and characterization of revertant cell lines. II. Growth control of a polyploid revertant line derived from SV40-transformed 3T3 mouse cells. J. Cell. Physiol., *82*: 93–100.

Risser, R., and R. Pollack 1974 A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology, *59*: 477–489.

Scher, C. D., and W. A. Nelson-Rees 1971 Direct isolation and characterization of "flat" SV40 transformed cells. Nature, New Biol., *233*: 263–265.

Shodell, M. 1972 Environmental stimuli in the progression of BHK/21 cells through the cell cycle. Proc. Nat. Acad. Sci., *69*: 1455–1459.

Smith, H., C. Scher and G. Todaro 1971 Induction of cell division in medium lacking serum growth factor by SV40. Virology, *44*: 359–370.

Todaro, G., and H. Green 1963 Quantitative studies on the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol., *17*: 299–313.

Todaro, G., H. Green and B. Goldberg 1964 Transformation of properties of an established cell line by SV40 and polyoma virus. Proc. Nat. Acad. Sci., *51*: 66–73.

Todaro, G., M. Matsuya, S. Bloom, A. Robbins and H. Green 1967 Stimulation of RNA synthesis and cell division in resting cells by a factor in serum. Growth regulating substances for animal cells in culture. Wistar Symposium Mono., *7*: 87–98.

Vogel, A., and R. Pollack 1973 Isolation and characterization of revertant cell lines. IV. Direct selection of serum revertant sublines of SV40-transformed 3T3 mouse cells. J. Cell. Physiol., *82*: 189–198.

Vogel, A., R. Risser and R. Pollack 1973 Isolation and characterization of revertant cell lines. III. Isolation of density-revertants of SV40-transformed 3T3 cells using colchicine. J. Cell. Physiol., *82*: 181–188.