

Isolation and Characterization of Revertant Cell Lines

II. GROWTH CONTROL OF A POLYPLOID REVERTANT LINE DERIVED FROM SV40-TRANSFORMED 3T3 MOUSE CELLS¹

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ABSTRACT 3T3 mouse fibroblasts in 10% calf serum cease to increase in number at confluence. SV40-transformed 3T3 cells lose their sensitivity to this growth control and so are able to reach multilayered cell densities. We previously described a polyploid revertant line F1SV101, isolated from a SV40-transformed cell, that has a low maximum cell density (Pollack et al., '68), despite the continued presence in this revertant of SV40-specific DNA, RNA and T-antigen. We have examined the mechanism by which the revertant maintains a low saturation density, and find that at confluence the revertant and 3T3 are both reduced in mitotic index, fraction of cells synthesizing DNA, and rate of DNA synthesis. The transformed cell does not respond to confluence. None of the lines sheds intact cells into the medium at confluence.

The growth curve of the normal mouse cell line 3T3 in 10% calf serum plateaus abruptly at confluence. The plateau is the result of a cessation of DNA synthesis by more than 95% of cells at that density (Nilausen and Green, '65). The growth curve of SV101, a subline of 3T3 that is transformed by the papova virus SV40, shows no plateau even at much higher cell densities (table 1). This response of 3T3 to confluence, called topo-inhibition (Dulbecco, '70) or contact-inhibition, has been accepted as an *in vitro* analog of the state of growth control found in normal tissues, and similarly the loss of contact-inhibition has been taken as an *in vitro* model of proliferation characteristic of tumor cells (Aaronson and Todaro, '70). Negative selection on a cloned population of SV40 transformed 3T3 cells permits the recovery of revertants (Pollack et al., '68; Ozanne et al., '70; Culp et al., '71; Culp et al., '72), whose growth curves once again display the plateau characteristic of 3T3. Revertant cells have been shown, by fluctuation analysis, to arise directly from transformed cells in the absence of selective agents (Pollack, '70). One revertant, F1SV101, has been studied in detail.

While F1SV101 and 3T3 both have a low saturation density (table 1), and share certain cell surface characteristics

(Pollack and Burger, '69; Mora et al., '71), F1SV101 is not identical to 3T3. F1SV101 cells are larger (Hough, P., personal communication) and have more chromosomes and more DNA per cell than either 3T3 or SV101 (table 1). Also, F1SV101 and SV101 both contain SV40-specific DNA, SV40-specific RNA (Ozanne et al., '73) and SV40-specific T-antigen (Pollack et al., '68). Both the revertant and SV101 will grow in medium containing depleted serum, but 3T3 will not (Jainchill and Todaro, '70). At confluence, both 3T3 and F1SV101, but not SV101, incorporate ³H-TdR at a much lower rate per cell than in sparse culture (Romano and Colby, '73). However, the plateaued growth curve of the revertant cells need not be solely the result of contact-inhibition of DNA synthesis. Revertant cells in contact may greatly extend all or part of the cell cycle. By coupling division with cell death or shedding, they may even continue to divide despite contact.

The nature of the growth control re-acquired by F1SV101 is the subject of this paper.

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TABLE 1
Growth parameters of mouse cell lines

Cell line	Doubling time (days)	Saturation density ($\frac{\text{cell}}{\text{cm}^2} \times 10^{-4}$)	Plating efficiency ($\frac{\text{colonies}}{\text{cells plated}}$)	Chromosomes per cell ¹ (median and range)	DNA per cell ² (G1 mode)
3T3	0.92	9	0.5	73 (75-81)	1.5
SV101	0.87	>45	0.3	74 (60-80)	1.5
F1SV101	1.20	7	0.5	118 (100-140)	3.5

Three established lines of mouse embryo origin were studied. 3T3 is a clone of the original 3T3 (Swiss) isolated in 1963.³ SV101 is a cloned SV40 transformant of 3T3, isolated in 1964.⁴ F1SV101 was isolated in 1968 from a high density culture SV101 by negative selection with FUdR.⁵ The three lines were subcloned approximately one month before these experiments. Cells were grown on 9.6-cm² plates (Falcon) in Dulbecco's modified Eagle's medium with 10% calf serum, 10⁻⁵ M thymidine, and 50 µg/ml Aureomycin. The complete medium was sterilized by pressure filtration (Millipore). Cultures were fed 1.5 ml of medium every three days. Stocks were carried at densities less than confluence in 29-cm² plates (Falcon).

¹ Pollack, R., S. Wolman and A. Vogel ('70).

² Assayed on Los Alamos Scientific Laboratory Flow Micro Fluorimeter (A. Vogel, H. Crissman and S. Cram, personal communication). DNA per cell expressed as multiple of the DNA per normal diploid mouse cell.

³ Todaro, G., and H. Green ('63).

⁴ Todaro, G., H. Green and R. Goldberg ('64).

⁵ Pollack, R., H. Green and G. Todaro ('68).

MATERIALS AND METHODS

Cell lines

Subclones of 3T3, SV3T3101 and F1SV101 were isolated approximately one month before these experiments began. Parameters of cell growth were measured simultaneously on all these lines (table 1).

Cell densities were divided into three classes: sparse, partly confluent, and fully confluent.

For sparse cultures, plates were inoculated at 10³ cells/cm² and used either at three days or, after a medium change on the third day, at six days.

For partly confluent cultures, plates were inoculated at 10⁴ cells/cm² and used after three days, when, for 3T3, most areas appeared confluent, but some mitoses were still visible near remaining small bare regions of the dish.

For fully confluent cultures, the medium was changed on partly confluent plates at three days and they were used at six days; cell counts of 3T3 and F1SV101 cultures showed no increase in cell density from the fifth to sixth day after inoculation.

Actual cell density was always measured at the beginning of each experiment by trypsinizing and counting 9.6-cm² plates.

Fraction of cells in mitosis

After exposure to 0.01 µg/ml Velban (Gibco) for two or three hours, attached

cells were trypsinized, pooled with cells floating in the medium, centrifuged, swelled in 0.38% KCl, fixed in methanol-acetic acid (3:1) overnight, recentrifuged and spread onto clean wet cold slides. The slides were stained with Giemsa, dehydrated, mounted and examined at × 400. At least 1000 nuclei were counted for each determination. The counts were normalized to fraction in mitosis/hour.

Incorporation of ³²P-phosphate into DNA

Plates containing cells at different densities were inoculated with carrier-free ³²P-phosphate at a final specific activity of 10 µCi/ml. At different times after inoculation, the cells were washed twice with cold PBS, dissolved in 2 ml of 1 N NaOH and incubated at 60° to hydrolyze RNA. After one hour, the samples were chilled and 2 ml of 1 N HCl and 4 ml of 20% TCA were added to precipitate the DNA, which was trapped on glass fiber filters and counted. In a reconstruction experiment with 10⁵ unlabeled cells, ³H RNA, and ³²P DNA, 100% of the ³²P DNA counts and fewer than 0.5% of the ³H-RNA counts were recovered in this procedure.

Incorporation of ³H-TdR

After exposure to 2.5 µCi/ml ³H-TdR for periods from 1 to 48 hours, cells in duplicate dishes were solubilized with 1 ml of 1 N NaOH and the DNA was precipitated with an equal volume of cold 20%

TCA. The precipitates were trapped on glass-fiber filters and counted. The rate of DNA synthesis at a given cell density was obtained from a plot of $^3\text{H-TdR}$ incorporated versus incorporation time.

At the time all lines were cloned, the medium was supplemented with 10^{-5} M nonradioactive thymidine (see cell lines, above). This carrier thymidine affected neither the doubling time nor the saturation density of any line but was essential for $^3\text{H-TdR}$ incorporation. In the absence of it, $^3\text{H-TdR}$ was incorporated into TCA-precipitable material for only 30 minutes, and initial rates of incorporation for a given cell line at a given cell density were not reproducible. However, in the presence of 10^{-5} M cold TdR, the labeled TdR was incorporated at a constant reproducible rate of 48 hours.

The ratio of the rate of incorporation to the specific activity decreased by only 10% when the added $^3\text{H-TdR}$ was increased from 2.5 $\mu\text{Ci/ml}$ to 20 $\mu\text{Ci/ml}$ (table 2). This result indicates that under experimental conditions, $^3\text{H-TdR}$ incorporation was effectively proportional to total thymidine incorporation, and that therefore thymidine was not a limiting metabolite, nor was the pool of thymidine appreciably changed by the addition of 2.5 $\mu\text{Ci/ml}$ $^3\text{H-TdR}$ to the medium.

Radioautography of $^3\text{H-DNA}$

Cells were inoculated into 9.6-cm² dishes containing sterile 12 mm circular glass cover slips (area = 1.13 cm²): After various periods of exposure to 2.5 $\mu\text{Ci/ml}$ $^3\text{H-TdR}$, cover slips were washed twice 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, stored at 4°C for

48 hours, developed in Dektol, and permitted to dry and harden overnight. The developed slides were then stained in hematoxylin (Harris), blued in LiCl_2 , cleared through alcohol to xylene, mounted in Permount, and photographed with Tri X film at $\times 500$, phase contrast (Zeiss). Silver grains were seen only overlying nuclei, which had been stained blue by the hematoxylin. Significant cytoplasmic localization of $^3\text{H-TdR}$, a sign of contamination by mycoplasma, was not observed.

RESULTS

Contact-inhibition of mitosis

The density-dependence of mitosis was determined from measurements of the fraction of cells in metaphase over a hundred-fold range of cell densities for each line. In sparse cultures of each line about four per cent of the cells entered mitosis each hour (table 3).

The mitotic index of 3T3 fell with increasing cell density until at confluence only 0.04% of 3T3 cells were dividing per hour, a residual value equal to 1% of that of sparse cultures (table 3). Most cells of the revertant line F1SV101 also ceased dividing at confluence. The mitotic index of F1SV101 at confluence was 3.3% of the mitotic index in sparse culture.

In contrast, the mitotic index for confluent SV101 was actually higher than for sparse cultures (table 3). The reason for this two-fold increase in SV101 mitotic index with increasing density is not known.

These data clearly indicate that unlike SV101 cells, F1SV101 cells have a marked inhibitory response to cell-cell contact. The mitotic-rate data rule out the possibility that F1SV101 cells proliferate at confluence at the same rate as they do in sparse culture.

TABLE 2
Rate of incorporation of $^3\text{H-TdR}$ by F1SV101 cells

Radioactivity added ($\mu\text{Ci/ml}$)	Thymidine available (μM)			Specific activity ($\mu\text{Ci}/\mu\text{M}$)	Rate of $^3\text{H-TdR}$ incorporation (cpm/ 10^4 cells/h)	Ratio of rate of incorporation to specific activity
	Radioactive	Carrier	Total			
2.5	0.125	10	10.12	0.247	125	506
10	0.5	10	10.5	0.955	425	445
20	1.0	10	11.0	1.81	825	455

¹ F1SV101 cells were at a density of 1.4×10^4 cells/cm² in this experiment.

TABLE 3

Mitotic fraction of cell lines at different cell density

Cell density (cell/cm ² × 10 ⁻⁴)	Per cent of cells in mitosis/hr (mitotic index)		
	3T3	SV101	F1SV101
Sparse (0.08–0.20)	3.7 ¹	4.2	5.4
Partly confluent (2.8–5.9)	0.08	—	0.48
Fully confluent (6.2–42)	0.04	7.6	0.18
Residual activity ²	1.1%	180%	3.3%

¹ Each value is the average of at least two measurements done on cultures at different densities within the stated ranges.

² Mitotic index at confluence as per cent of rate in sparse culture.

Density-dependence of DNA synthesis

In order to examine whether the decreased mitotic index of the revertants was accompanied by a decrease in the rate of DNA synthesis at confluence, we first measured the rate of incorporation of ³²P into DNA. Cultures of 3T3 and F1SV101 showed a clear inhibitory response to confluence, while SV101 failed to respond (table 4). The residual rates of incorporation (7% for 3T3 and 11% for F1SV) were somewhat higher than the residual rates of mitosis (table 3).

We next measured the rate of incorporation of ³H-TdR at different cell densities. Sparse cultures of all three lines had high rates of ³H-TdR incorporation. SV101 cells kept up this rate of incorporation, showing an increase in ³H-TdR incorporation rate at very high densities (table 4). This increase, like the increase in SV101 mitotic rate, was unexpected and the reason for it is not known.

At confluence both 3T3 and F1SV101 showed a decrease in ³H-TdR incorporation: confluent rates were 2.1% and 3.0% of the sparse rates, respectively (table 5). These residual rates were lower than the

residual rates obtained with ³²P, and were very similar to the residual mitotic rates (table 3).

Transport variations between cells growing at different rates (Romano and Colby, '73) and variations in pool size prevent us from concluding whether the ³H or the ³²P rates are the "true" residual rates of DNA synthesis. However, these data coupled with the mitotic index data are sufficient to permit the conclusion that 3T3 and F1SV101 respond to confluence by a marked decrease in proliferation, while SV101 does not.

It must be stressed that, while 3T3 cells at confluence are known to be unable to progress past the beginning of G1 (Nilausen and Green, '65) the data presented here do not permit the conclusion that confluent F1SV101 are blocked there or at any other single part of the cell cycle.

Radioautographic assay of the fraction of cells synthesizing DNA

F1SV101 and 3T3 cultures respond to confluence by reducing DNA synthesis and mitosis to about 3% of the rate of sparse cultures. SV101 cells have neither inhibitory response. This lower rate of

TABLE 4

Incorporation of ³²P-phosphate into DNA

Cell density (cell/cm ² × 10 ⁻⁴)	cpm ³² P-phosphate/h/10 ⁴ cells		
	3T3	SV101	F1SV101
Sparse (0.10–0.28)	88 ¹	114	229
Partly confluent (0.28–2.6)	47	108	93
Fully confluent (3.4–10.7)	6.1	109	26
Residual activity ²	6.9%	95.6%	11.3%

¹ Each value is the average of at least two measurements done on cultures at cell densities within the stated ranges. For each measurement, cell density was obtained by coulter-counter assay of a trypsinized sister plate.

² Rate at confluence as per cent of rate in sparse culture.

TABLE 5
Rate of incorporation of $^3\text{H-TdR}$ by cell lines
at different cell densities

Cell density ¹	cpm $^3\text{H-TdR}/\text{H}/10^4$ cells		
	3T3	SV101	F1SV101
Sparse	108	90	448
Partly confluent	22	130	39.3
Fully confluent	2.3	295	13.3
Residual activity ²	2.1%	326%	3.0%

¹ See footnote 1, table 4.

² Rate at confluence as per cent of rate in sparse culture.

DNA synthesis may be due to a minority of cells synthesizing DNA at the maximum rate, or to all of the cells synthesizing DNA at a 30-fold reduced rate, or to some intermediate condition. Radioautography of incorporated $^3\text{H-TdR}$ was employed to determine the fraction of cells synthesizing DNA.

When sparse cultures of each line were pulsed with $^3\text{H-TdR}$ for 1 to 48 hours, the shortest pulse showed the instantaneous fraction of cells in S to be about 0.35 (table 6), suggesting that DNA synthesis (S) occupied about 35% of the cell cycle, or seven to nine hours (table 1). For 3T3, this value is in reasonable agreement with previous studies (Nilausen and Green, '65).

After four hours exposure to $^3\text{H-TdR}$, about half the cells in a sparse culture incorporated the label, and after one day, all cells of all three lines synthesized DNA (table 6).

In contrast, when confluent F1SV101 were pulsed for various times with $^3\text{H-TdR}$, a full 48 hour exposure was required to label 62% of the cells (table 6). This value, which is in excellent agreement with a previous study on F1SV101 (Scher and Nelson-Rees, '71), indicates that the fraction of cells entering S per hour at

confluence is at most one-fifth the fraction of cells entering S per hour in sparse cultures of F1SV101, 3T3 or SV101 (table 6).

To determine the fraction of cells incorporating $^3\text{H-TdR}$ at different cell densities, cultures were radioautographed after four-hour pulses. This duration of exposure resolved the fraction in S (table 6) at any density while permitting enough incorporation for clear determination of labeled nuclei (fig. 1).

3T3 cultures responded to confluence by reducing the fraction of cells incorporating $^3\text{H-TdR}$ (fig. 1a). At confluence the 3T3 fraction in S was about 5% of the fraction in S in sparse culture (table 7). For 3T3, the fraction in M at confluence is only 1% of the fraction in M in sparse culture (table 3), so radioautography suggests that most (four-fifths) of the 3T3 cells which synthesized DNA in confluence did not enter mitosis. What happens to this small fraction (about 4%) of confluent 3T3 cells is not known. Perhaps, as with certain serum-transformed "flat" cells (Scher and Nelson-Rees, '71), they die, providing an equilibrium between DNA synthesis and cell death.

Confluence did not affect the rate at which SV101 cells entered S. About half the cells incorporated $^3\text{H-TdR}$ in four hours at any cell density measured (fig. 1a). The increase in rate of incorporation of $^3\text{H-TdR}$ by SV101 (table 5) is not matched by an increase in the fraction in S (table 7).

Confluence caused the majority of F1SV101 cells to cease entering S (table 7). At confluence F1SV101, like 3T3, had a higher residual fraction of cells in S than in M (table 3). The fraction of F1SV101 in S was 29% of the fraction in sparse culture, while the fraction entering M

TABLE 6
Radioautography of cultures after different periods of exposure to $^3\text{H-TdR}$

Cell line	Cell density (cells/cm ² × 10 ⁻⁴)	Fraction of cells incorporating $^3\text{H-TdR}$ after exposure for hours					
		1	4	8	24	32	48
3T3	2.2	0.36	0.52	—	1.0	1.0	1.0
SV101	0.85	0.35	0.62	0.92	1.0	—	1.0
F1SV101	0.7	0.40	0.51	—	1.0	1.0	1.0
	5.2	0.22	0.27	0.39	0.42	—	0.62

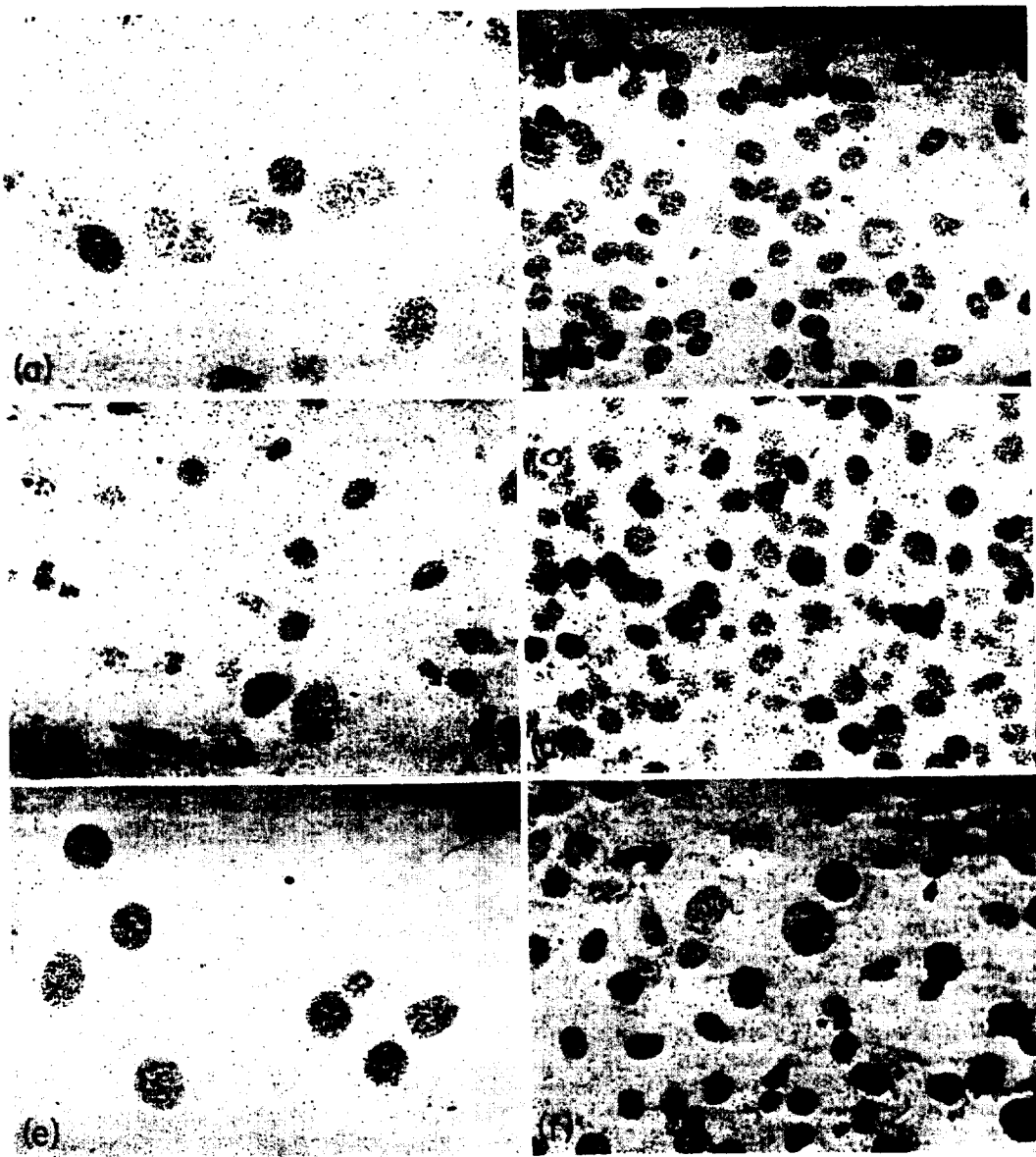


Fig. 1 Radioautography of mouse cell lines after exposure to $^3\text{H-TdR}$ for four hours. (a) 3T3, sparse; (b) 3T3, fully confluent; (c) SV101, sparse; (d) SV101, fully confluent; (e) F1SV101, sparse; (f) F1SV101, fully confluent. The fraction of nuclei labeled falls markedly at confluence in 3T3 (a, b), to a lesser extent in F1SV101 (e, f) and not at all in SV101 (c, d). Density in cells/cm $^2 \times 10^4$: (a) 1.9, (b) 10.5, (c) 2.8, (d) 26.5, (e) 0.85, (f) 5.2.

was 3% of the fraction in sparse culture. Therefore, the residual fraction in S was almost ten times higher than the residual fraction in M, so almost every one of the 15% of confluent F1SV cells that entered S failed to enter M.

DISCUSSION

The responses of 3T3 and F1SV to confluence are quite similar: a majority of cells in both lines do not enter M, do not enter S, and for both lines DNA synthesis

TABLE 7

Radioautography after four-hour exposure to $^3\text{H-TdR}$ of cultures at different cell densities

Cell density ¹	Fraction of nuclei labeled ²		
	3T3	SV101	F1SV101
Sparse	0.52	0.69	0.51
Partly confluent	0.44	0.62	0.38
Fully confluent	0.025	0.62	0.15
Residual activity ³	4.8%	89%	29%

¹ See footnote 1, table 3.

² At least 500 nuclei were scanned on each radioautograph cover slip. Three or more experiments were performed for each density category. Exact cell density was determined before each experiment by counting a sister culture. See METHODS section.

³ Fraction at confluence as per cent of fraction at log density.

per culture is much reduced. Whether the minority of cells of each line that incorporate DNA in confluence without entering mitosis die before M, are trapped in S, or are traversing the cell cycle at an extremely slow rate, remains to be determined.

Scher and Nelson-Rees (71) have described in detail a class of Balb-3T3 lines selected directly for their ability to grow in depleted serum acquired as a result of SV40 infection. A minority of these lines have low saturation densities and some are, like F1SV101, polyploid. However, these "flat-transformants" are not derived from dense-transformants as was F1SV101, so there is no reason to expect the properties of revertants and flat-transformants to be identical. For example, 48% of the cells of one flat transformant line underwent mitosis in confluence after 60 hours, as measured by time-lapse cinematography. This is equivalent to 0.8% mitosis per hour, which is about $5 \times$ higher than the rate observed here for F1SV101 at confluence (table 3). While we confirm their radioautographic data for F1SV101, we have obtained different results with SV101 than those Scher and Nelson-Rees published for a similar transformed Balbc/3T3 line, "11-8." Only 76% of the cells of "11-8" were labeled by radioautography even after 48 hours exposure to $^3\text{H-TdR}$, while 92% of SV101 cells are labeled after only eight hours exposure to $^3\text{H-TdR}$.

We attempted to recover shed cells

from the medium, but found very few intact cells in the medium of any line at any cell density. Shed cells never exceeded 2% of the cells on the dish per day. While the possibility remains that some dead cells were shed, it seems clear from the data presented here that the low saturation density of F1SV101 and 3T3 cannot be due to a balance between continued DNA synthesis at the rate found in logarithmically growing cells, and cell death.

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