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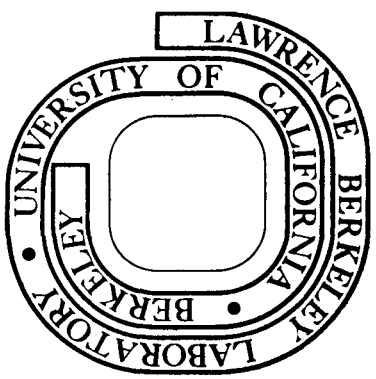

DISCLAIMER

EFFECT OF SERUM ON THE GROWTH OF BALB 3T3 A31 MOUSE FIBROBLASTS AND AN SV40-TRANSFORMED DERIVATIVE

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ABSTRACT

The effect of serum on the growth properties of non-transformed Balb 3T3 A31 and SV40-transformed Balb 3T3 A31 was studied. The concentration of serum in the growth medium of non-transformed cells had little effect on the initial population doubling time, but did regulate the cell density at which the population became quiescent in G_1. The doubling time of transformed cells, however, was increased significantly as the concentration of serum was decreased below 4%. This effect on the growth of transformed cells was seen at serum concentrations so low that non-transformed cells did not complete one population doubling. Flow microfluorimetric analysis of these populations indicated that the primary effect of different serum concentrations on the non-transformed cells was to modulate the average residence time in G_1; whereas, all the cell cycle phases of the transformed cells were affected by serum. At saturation densities, the non-transformed cells became quiescent in G_1, but the transformed cells still traversed the cell cycle and their saturation density appeared to be a balance between cell production and cell death occurring primarily in the G_1 phase of the cell cycle.

INTRODUCTION

The growth properties of mammalian cells in culture are thought to reflect the corresponding properties in vivo (1, 22). Cell lines that have "tight" growth regulatory properties in culture generally do not give tumors when injected back into animals (1). Balb 3T3 A31 mouse fibroblast cells exhibit "tight" growth control in culture, but when transformed by SV40 virus their growth potential in culture under a given set of conditions increases as does their ability to form tumors in animals (1). These observations suggest that an understanding of the mechanisms for the regulation of growth of mammalian cells in culture will be relevant to growth control in vivo.

One of the elements that has been shown to play a roll in the regulation of growth of mammalian cells in culture is serum. Holley and Kiernan (9) found that the concentration of serum in the growth medium determined the final cell density of both transformed and non-transformed mouse 3T3 cells in culture. This final cell density or saturation density was much higher for transformed cells than non-transformed cells at a given serum concentration. Nilausen and Green (14) demonstrated that when non-transformed mouse fibroblasts reached their saturation density cell cycle traverse was arrested in G_1. This block, however, is reversible and cell cycle traverse can be stimulated by the addition of fresh serum (25). Factors other than serum concentration can regulate cell growth by restricting passage through G_1 (17,20,24) and it has been postulated that the growth of non-transformed cells is controlled by a restriction point in G_1 that is sensitive to suboptimal growth conditions (15).

The effect of limitation of serum or other medium components on the growth of transformed cells has not been clearly defined. There is some evidence that as a population of transformed cells reaches its saturation

density, and presumably depletes some medium components, the mitotic index drops (6). Paul (11) has demonstrated that with SV3T3 cells low levels of leucine inhibit cell cycle traverse; but, in contrast to non-transformed cells, the transformed cells were not arrested in one phase of the cell cycle.

In this report we describe the effect of serum on the doubling time of Balb 3T3 A31 and a closely derived SV40 virus transformed line, FNE. Our results indicate that limitation of serum has less effect on the initial population doubling time of Balb 3T3 A31 than on FNE. Furthermore, serum regulates the growth of Balb 3T3 A31 by increasing the average residence time in G_1, but regulates FNE growth by lengthening the average residence time of all phases of the cell cycle.

MATERIALS AND METHODS

Cell Culture Techniques

The cells used in this study were obtained from Helene Smith of the Naval Biomedical Laboratory, Oakland, California. Balb 3T3 A31 was cloned prior to use in these experiments to give the derivative, Balb 3T3 A31 HYF. FNE is a derivative of Balb 3T3 A31 which has been transformed with SV40 virus. It is producing SV40 T-antigen and unlike Balb 3T3 A31 is highly tumorigenic when injected as a suspension in newborn Balb/c mice[1]. It was passaged nineteen times as described below before use in these experiments.

Both cell lines were carried in 100 mm plastic dishes (Falcon, Oxnard, Calif.) and incubated in a 10% CO_2 incubator at 37°. The cells were grown in Vogt and Dulbecco's modification of Eagle's medium (27), containing 10% newborn calf serum (GIBCO, Santa Clara, Calif.). The cells were transferred

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[1] Smith, H., personal communication.

when reaching confluency by removal from the dishes with 0.05% trypsin (Difco, 1:250; Detroit, Mich.) in 25 mM Tris buffer, pH 7.4 containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4 (isotonic Tris buffer). The seeding density was 1/10 of the saturation density.

Growth Curves

The growth of the cultures in media containing different concentrations of calf serum was monitored by determining the increase in the number of cells per culture dish as a function of time. On day 0 four 35 mm plastic dishes were seeded for each time point to be taken. The dishes were seeded with 5.0×10^4 cells in 1.5 ml of test medium. At each time point the cells were removed from the dishes by treatment with 2.0 ml 0.01% trypsin in isotonic Tris buffer for 20 min at 37°. The number of cells per dish was determined by counting an aliquot of the trypsin solution in a Coulter counter model Fn (Coulter Electronics, Hialeah, Florida). The values were corrected for coincidence counting and averaged.

Autoradiography

To determine the percentage of the population in S at each time point, the cultures were pulsed for 15 min with 1 uCi/ml ^{3}H-thymidine (20.1 Ci/mM; New England Nuclear, Boston, Mass.). At the end of the pulse the cells were washed on the dish 2 times in isotonic Tris buffer, swollen with 0.075 M KCl and fixed with methanol-acetic acid (3:1)(19). After air drying the dishes were coated with Kodak Nuclear Track Emulsion NTB-2 (Eastman Kodak, Rochester, N.Y.), exposed in the dark at room temperature for 14 days, developed, and fixed. The cells were stained with crystal violet and the percentage of labeled nuclei was determined by counting a total of 2000 cells for each time point.

Flow Microfluorimetry

The amount of DNA per individual cell was quantified by staining the cells with acriflavine (26) and measuring the amount of fluorescence per cell by flow microfluorimetry (10). These measurements were done in collaboration with Drs. Joe Gray and Marvin Van Dilla of the Lawrence Livermore Laboratory. In this technique, the stained cells are passed individually through the beam of an argon-ion laser (Spectra-Physics, Mountain View, Calif.) tuned to 488 nm. The pulse of fluorescent light was filtered to reduced scattered light and detected by a photomultiplier tube positioned at right angles to the laser beam. The resulting signal is amplified electronically and recorded in the memory of a pulse height analyzer (Northern Scientific, Middletown, Wisconsin). As the data accumulates it generates a histogram as shown in Figure 1. The data was stored on magnetic tape and processed by a program written for a Sigma 2 computer (Xerox, Rochester, N.Y.).[2] As seen in Figure 1 the integration of these plots gives the percentage of cells in the population in G_1, S, and G_2 plus M(G_2+M). The procedure used to obtain the proportion of the population in each of the phases is described in a separate report (2).

RESULTS

Effect of Serum on the Growth of Balb 3T3 A31 HYF and FNE

Figure 2 shows that the growth of Balb 3T3 A31 HYF and FNE is dependent on the concentration of serum in the medium. As the serum concentration increased, the doubling time of the population decreased (Table 1) and the saturation density increased. The doubling times in Table 1 were computed from the cell counts using a least squares curve fit program. Only values

[2] Wong, S., K. Wiley and A. Salmon, unpublished procedure.

that had correlation coefficients greater than 0.97 are reported. The initial doubling time of Balb 3T3 A31 HYF at serum concentrations below 8% was difficult to determine because of the small amount of growth seen with these serum concentrations, and above 8% the effect of serum concentration on the initial doubling time was minimal. The doubling time of FNE, on the other hand, was clearly dependent on the concentration of serum in the medium. Accurate population doubling times for FNE could be measured at serum concentrations as low as 1%. A comparison of the doubling times of Balb 3T3 A31 HYF and FNE at serum concentrations above 8% indicates that the two cell lines have the same minimum doubling time. Throughout the course of these experiments, the cultures were monitored microscopically for cells floating in the medium as an indication of cell death. The only evidence that cell death was occurring was with FNE after it had reached its saturation density.

Cell Cycle Distributions After Two Days Growth in Medium Containing Different Serum Concentrations

Flow microfluorimetry (FMF) can determine the distribution of a population in the cell cycle by measuring the DNA content of individual cells. Cells that are in G_1 have half the amount of DNA of cells that are in G_2 or mitosis. Cells that are in S have DNA contents between that of G_1 cells and G_2 cells depending on how far through S they have progressed. Assuming a random distribution of cells around the cell cycle, the proportion of the cells in a particular phase of the cell cycle is a function of the average residence time in that phase. Using the doubling time of the population and the distribution of cells in the various phases, the average

residence time of each phase can be calculated (12). The technique assumes a negligible death rate which appears to be true for Balb 3T3 A31 HYF and FNE growing logarithmically (see above). We have used this procedure to determine how different serum concentrations in the growth medium affect the average residence time of the two populations in the individual phases of the cell cycle.

The FMF distributions of Balb 3T3 A31 HYF after two days of growth in medium containing different serum concentrations are shown in Fig. 3. It was not possible to analyze the cells before day 2 because the cell cycle distributions were still affected by the state of the population prior to setting up the experiment (3). Table 2 gives the values for the integrated areas under the DNA histograms. These calculations gave values for the percentage of the population in S that agreed within 5% of those obtained by incorporation of a 15 min pulse of ^{3}H-thymidine followed by autoradiography to detect DNA synthesizing cells. As the serum concentration decreased below 8% the percentage of the population in G_1 increased while the other phases decreased. At low serum concentrations there were essentially no cells in S. ^{3}H-thymidine incorporation followed by autoradiography of cells growing in medium containing less than 2% serum confirmed that less than 3% of the cells were synthesizing DNA on day 2.

There appeared to be some cells with a DNA content of G_2+M cells even at low serum concentrations. It should be pointed out, however, that binucleated or aggregated cells would be recorded as having a DNA content equivalent to G_2+M cells. However, if aggregation or multinucleation were affecting the results, peaks representing higher multiples of G_2+M should be present in the histograms shown in Fig. 3. The absence of these peaks suggests that the G_2+M peak is not contaminated with a significant amount of aggregates or multinucleates.

The growth of Balb 3T3 A31 HYF was logarithmic on day 2 only in medium containing 8% serum or greater. Using the doubling time of 16 hr for these logarithmic populations (Table 1), the average residence time of cells in each of the cell cycle phases was calculated for the different growth conditions. These values are given in Table 2 and indicate that although the doubling times were identical for a range of serum concentrations, the average residence times varied. As the serum concentration increased, the value for G_1 decreased and the value for S increased. The average residence time for G_2+M remained approximately constant.

Figure 4 gives the FMF distributions of FNE growing in medium with different concentrations of serum. Integrations of the areas under the histograms gave the values in Table 3 for the distribution of cells in the cell cycle. Unlike Balb 3T3 A31 HYF, FNE even in medium with very low serum concentrations had a significant number of cells in all phases of the cell cycle. As an example, approximately 10% of the population was in S when the serum concentration was only 0.1%. This was confirmed by ^{3}H-thymidine incorporation followed by autoradiography. As seen in Fig. 2, FNE grew logarithmically at serum concentrations as low as 1%. When the average residence time of the cell cycle phases was calculated for the logarithmic populations it was apparent that as the doubling time increased the length of all phases increased.

Cell Cycle Distributions at Saturation Density

Using FMF we analyzed the cell cycle distributions of cultures of Balb 3T3 A31 HYF and FNE at their saturation density in medium containing different concentrations of serum. Balb 3T3 A31 HYF was predominately (>93%) in G_1 at saturation density regardless of the concentration of serum in the medium. ^{3}H-thymidine incorporation followed by autoradiography

indicated that less than 0.5% of the population was making DNA at any of the serum concentrations. FNE gave cell cycle distributions at saturation densities in media with different levels of serum as seen in Fig. 5. Even though the cultures had stopped increasing in density there were still cells in all parts of the cell cycle. The cells were were still incorporating ^{3}H-thymidine into DNA at all serum concentrations.

As mentioned above when cultures of FNE reached saturation density some cells could be seen in the medium. These cells were not viable when replated into fresh medium and when analyzed using the FMF technique they had DNA contents equivalent to G_1 cells.

DISCUSSION

The doubling time of a population of cells is dependent on three variables: (a) the length of the cell cycle; (b) the fraction of the population traversing the cycle; and (c) the rate of cell loss (4). A number of models have been proposed using these variables to explain the growth properties of mammalian cells. Prescott (18) has suggested that the growth rate of cells in culture is determined primarily by the length of G_1. Smith and Martin (21), on the other hand, have recently proposed that the doubling time of a population in a given environmental situation is dependent on the probability of cell cycle transit. Both of these models propose that the length of S, G_2 and M are constant, despite the evidence that *in vivo* at least the length of S and G_2 does vary (5, 11).

Understanding the mechanism of growth control becomes particularly important when considering malignant cells. Analysis of the growth properties of malignant cells *in vivo* suggests that they do not necessarily grow with a shorter doubling time than normal cells, but appear to be able to grow under conditions where normal cells will not (11). Under such

conditions the fraction of proliferating cells (13) is high in the case of malignant cells, but low with normal cells.

In the experiments reported here we have analyzed the growth properties of Balb 3T3 A31 HYF, a non-transformed mouse fibroblast with low tumorigenic potential, and a highly tumorigenic derivative, FNE, in media containing growth limiting amounts of serum. As seen by Holley and Kiernan (8) serum concentration primarily affects the cell density at which a population of non-transformed cells becomes quiescent. This stringent effect on the saturation density makes analysis of the effect of serum on the rate of population growth difficult. However, in other experiments (3) we have used a G_1 synchronized population to show that the level of serum in the medium has little effect on the time of transit from G_1 through S to G_2 and M, but does have an effect on the fraction of the population capable of being stimulated. These experiments suggest that the primary effect of serum on the growth of Balb 3T3 A31 HYF is in determining the fraction of the population capable of cell cycle transit. A secondary effect of serum may be on other variables determining the growth properties of the population, but these effects must be small and are difficult to separate from the primary effect of serum. These experiments are consistent with the model of Smith and Martin (21) and suggest that serum acts by determining the probability of cell cycle transit.

The doubling time of FNE, on the other hand, is very much dependent upon the concentration of serum in the medium at concentrations of serum below 4% (Table 1). These effects do not seem to be due to cell loss since microscopic observation of the cultures did not reveal a significant increase in floating dead cells as the doubling time increased. At concentrations of serum where the doubling time is lengthened the average residence time in each of the cell cycle phases is also lengthened (Table 3). As the

concentration of serum in the medium dropped to 0.4% or lower, the population accumulated in G_2+M. It is important to note that these effects of serum on the length of individual phases of the cell cycle of FNE were seen at serum concentrations so low that Balb 3T3 A31 HYF did not grow significantly. Thus, it is possible that serum may affect the average residence time of Balb 3T3 A31 HYF in the cell cycle phases other than G_1, but at concentrations where this effect may occur the population has become quiescent in G_1.

The cell cycle distributions at the saturation density of Balb 3T3 A31 HYF indicated that no matter what the concentration of serum in the medium, the cells stopped growing by becoming quiescent in G_1. FNE, on the other hand, did not become quiescent at its saturation density, but continued to traverse the cell cycle. Microscopic observations indicated that at saturation density dead floating cells were accumulating. FMF analysis of the floating cells indicated that death had occurred predominately in G_1.

These studies indicate that at serum concentrations where Balb 3T3 A31 HYF can grow significantly there is little effect of serum on the doubling time of the population. The primary effect is on determining the saturation density at which the population becomes quiescent. When this cell line is transformed with SV40 virus, the transformant grows at lower serum concentrations, but with doubling times that are dependent on serum concentration. These longer doubling times are the result of lengthening the average residence time in each of the cell cycle phases.

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Table I.

Effect of Serum on the Population Doubling Time of Balb 3T3 A31 HYF and FNE

% Serum	Doubling Time (hr)* Balb 3T3 A31 HYF	FNE
1.0	-	51
2.0	-	29
4.0	-	20
6.0	-	16
8.0	16	16
10.0	16	16
16.0	16	16
20.0	16	-

* The doubling times were computed from the cell counts using a least squares fitting program (see text).

Table II.

Effect of Serum on the Cell Cycle Distribution of Balb 3T3 A31 HYF

	G_1		S		G_2+M	
% Serum	% of Population	Average Residence Time (hr)	% of Population	Average Residence Time (hr)	% of Population	Average Residence Time (hr)
0.4	96.9	-	0	-	3.1	-
1.0	93.7	-	1.7	-	4.6	-
2.0	95.6	-	0	-	4.4	-
4.0	80.1	-	6.1	-	12.8	-
6.0	59.5	-	18.0	-	22.4	-
8.0	50.9	6.8	24.1	4.0	25.0	5.2
10.0	48.6	6.4	28.9	4.9	22.5	4.7
16.0	42.8	5.6	31.0	5.0	26.2	5.4
20.0	38.6	5.0	38.2	6.2	23.2	4.8

Table III.

Effect of Serum on the Cell Cycle Distribution of FNE

	G_1		S		G_2+M	
% Serum	% of Population	Average Residence Time (hr)	% of Population	Average Residence Time (hr)	% of Population	Average Residence Time (hr)
0.1	34.6	-	9.6	-	55.9	-
0.4	49.8	-	10.0	-	40.2	-
1.0	49.6	21.0	22.6	12.0	27.8	18.0
2.0	47.7	11.4	28.1	8.5	24.2	9.1
4.0	56.6	9.6	22.3	4.9	22.2	5.5
6.0	45.3	5.9	25.9	4.2	28.8	5.9
8.0	49.1	6.6	27.5	4.5	23.5	4.9
10.0	50.0	6.6	24.7	4.2	25.3	5.2
16.0	46.8	6.2	27.1	4.5	26.1	5.3

FIGURE LEGENDS

Figure 1. Idealized DNA histogram obtained by analyzing a non-synchronous population.

Figure 2. Growth of Balb 3T3 A31 HYF and FNE in medium containing different concentrations of new born calf serum.

Figure 3. DNA histograms of populations of Balb 3T3 A31 HYF harvested two days after seeding in medium containing the indicated serum concentration.

Figure 4. DNA histograms of populations of FNE harvested two days after seeding in medium containing the indicated serum concentration.

Figure 5. DNA histograms of populations of FNE harvested six days after seeding in medium containing the indicated serum concentration.

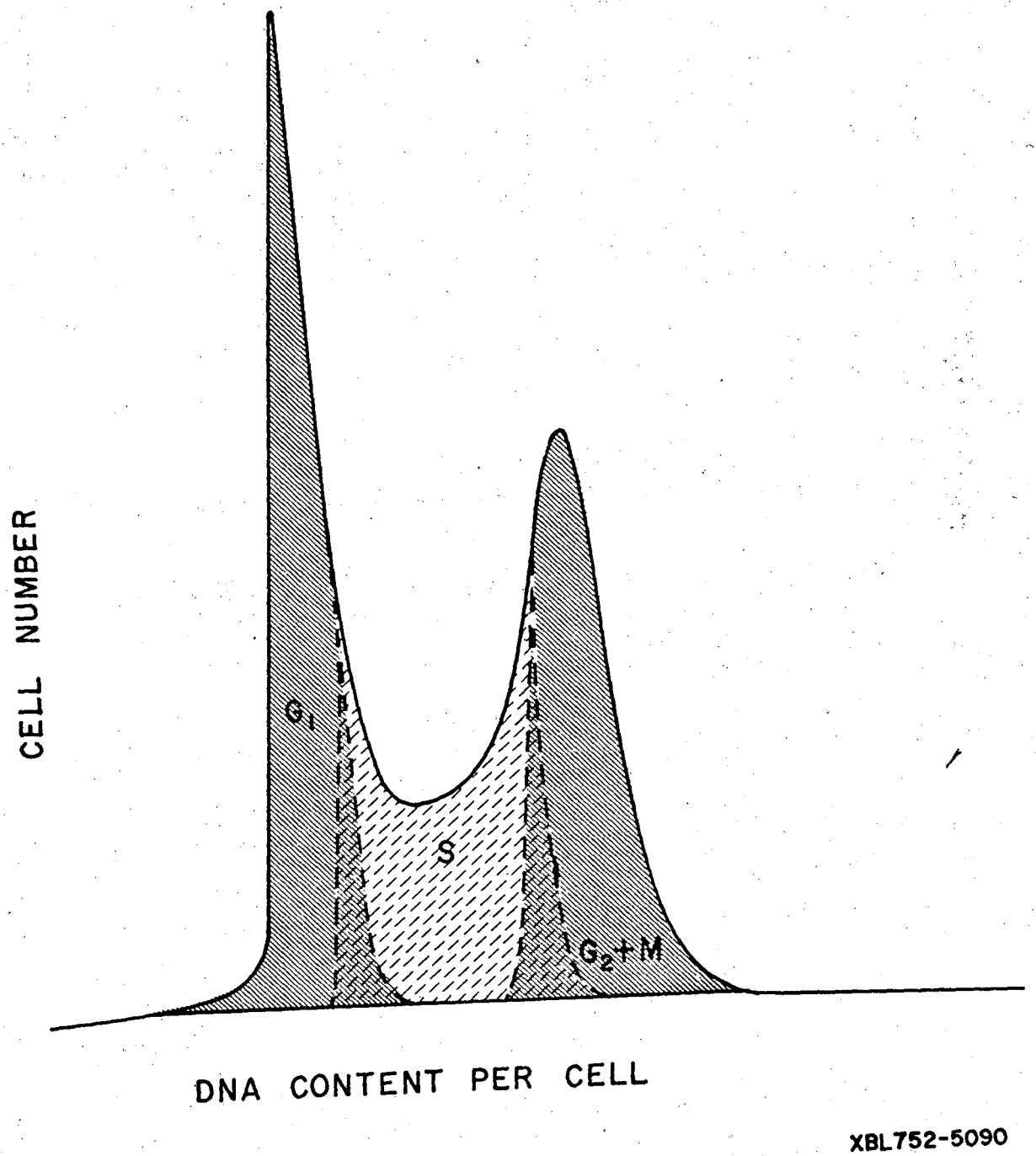

Fig. 1

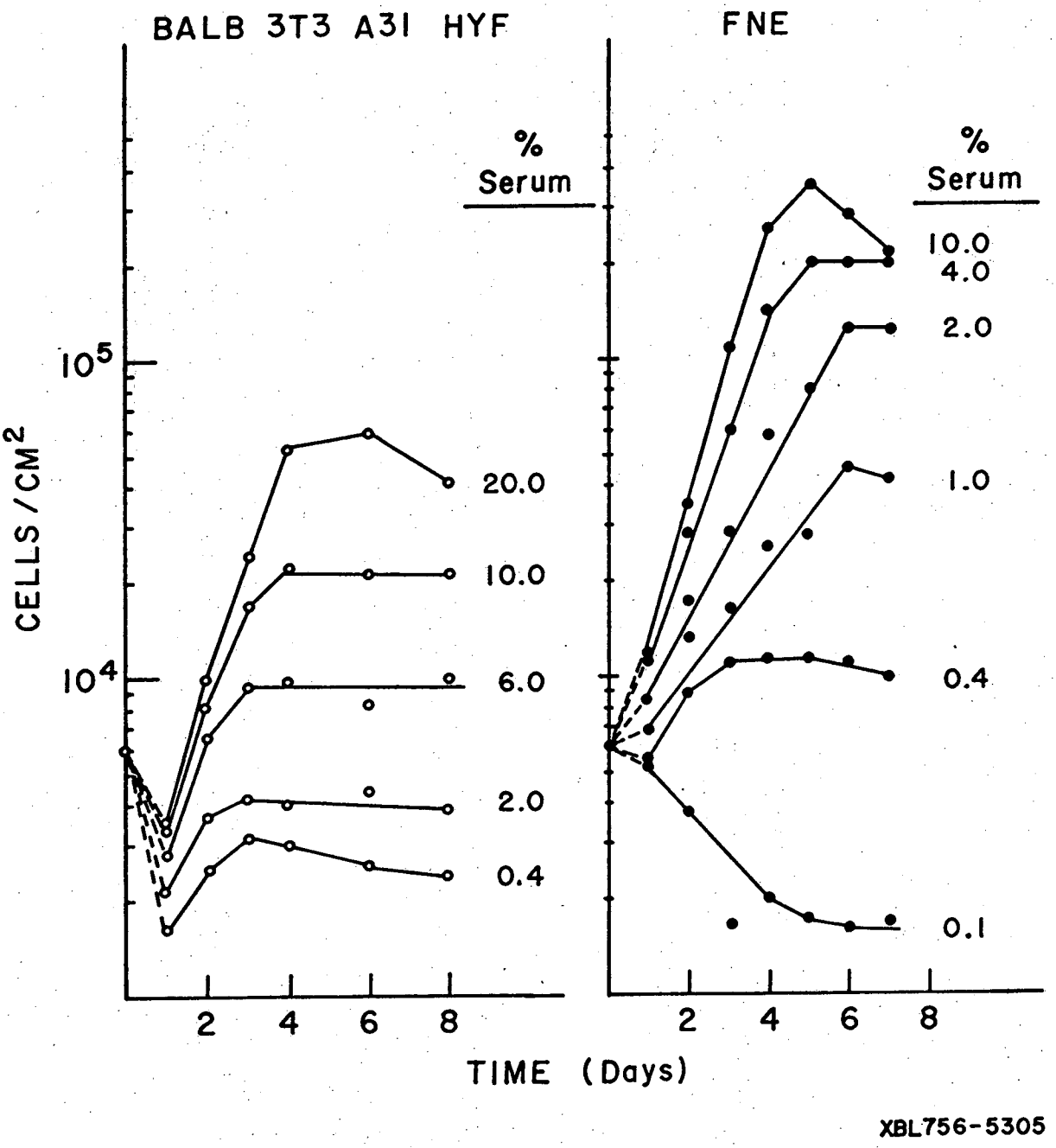
BALB 3T3 A31 HYF
FNE
% Serum
% Serum
10.0
4.0
2.0
1.0
0.4
0.1
20.0
10.0
6.0
2.0
0.4
10^5
10^4
CELLS / CM^2
2
4
6
8
TIME (Days)
XBL756-5305

Fig. 2

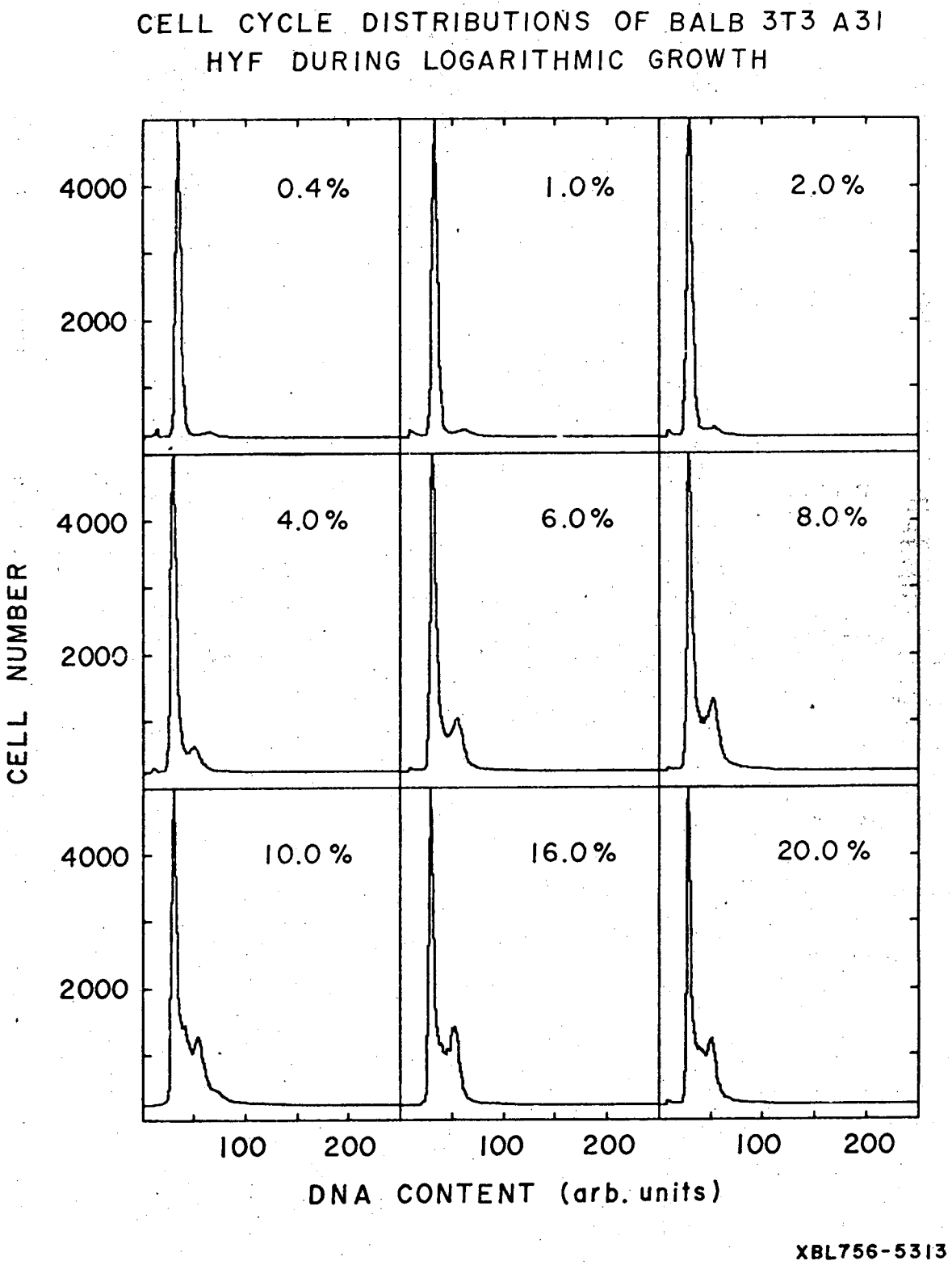

Fig. 3

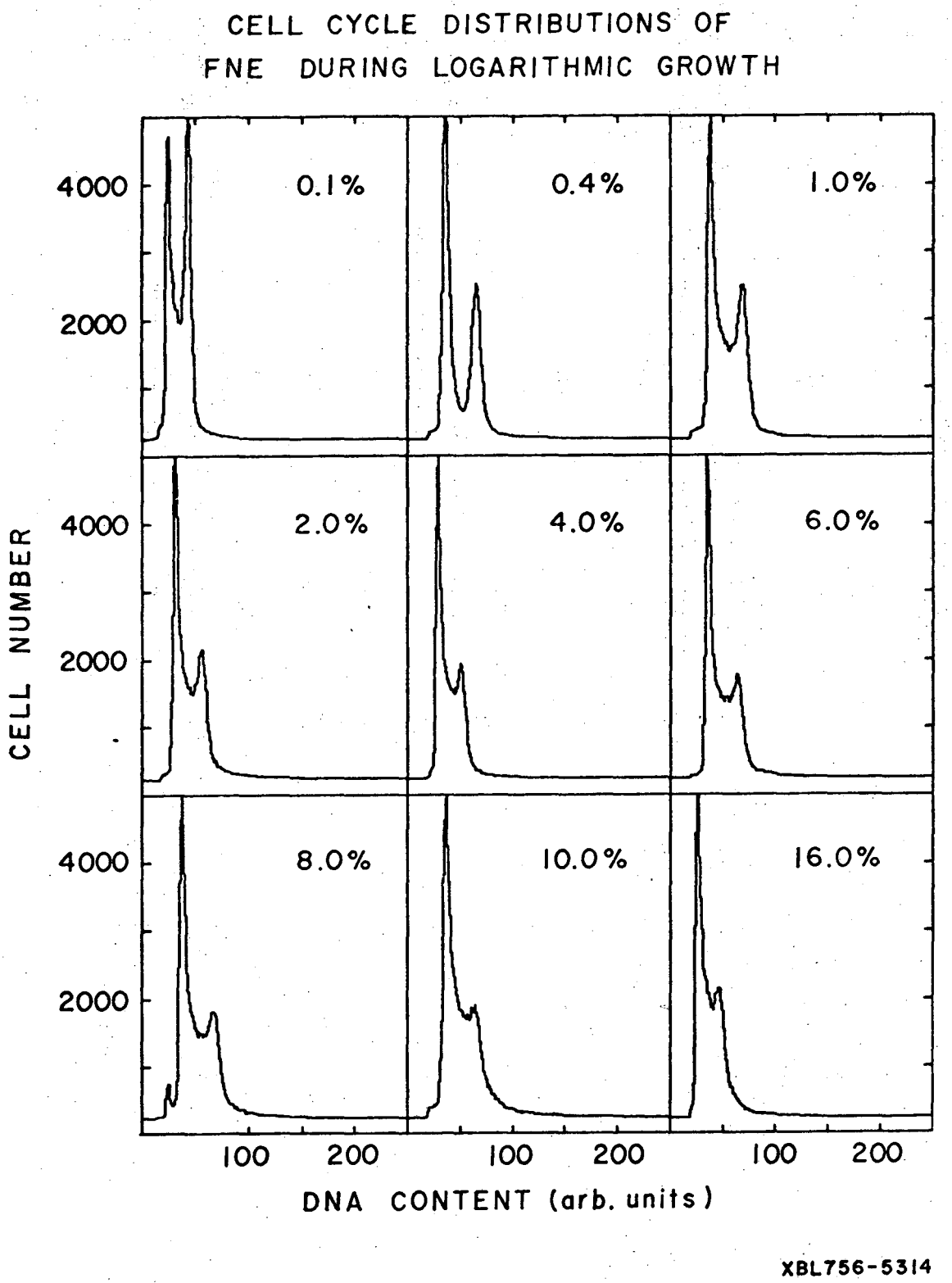
CELL CYCLE DISTRIBUTIONS OF
FNE DURING LOGARITHMIC GROWTH
0.1%
0.4%
1.0%
2.0%
4.0%
6.0%
8.0%
10.0%
16.0%
4000
2000
CELL NUMBER
100
200
DNA CONTENT (arb. units)
XBL756-5314

Fig. 4

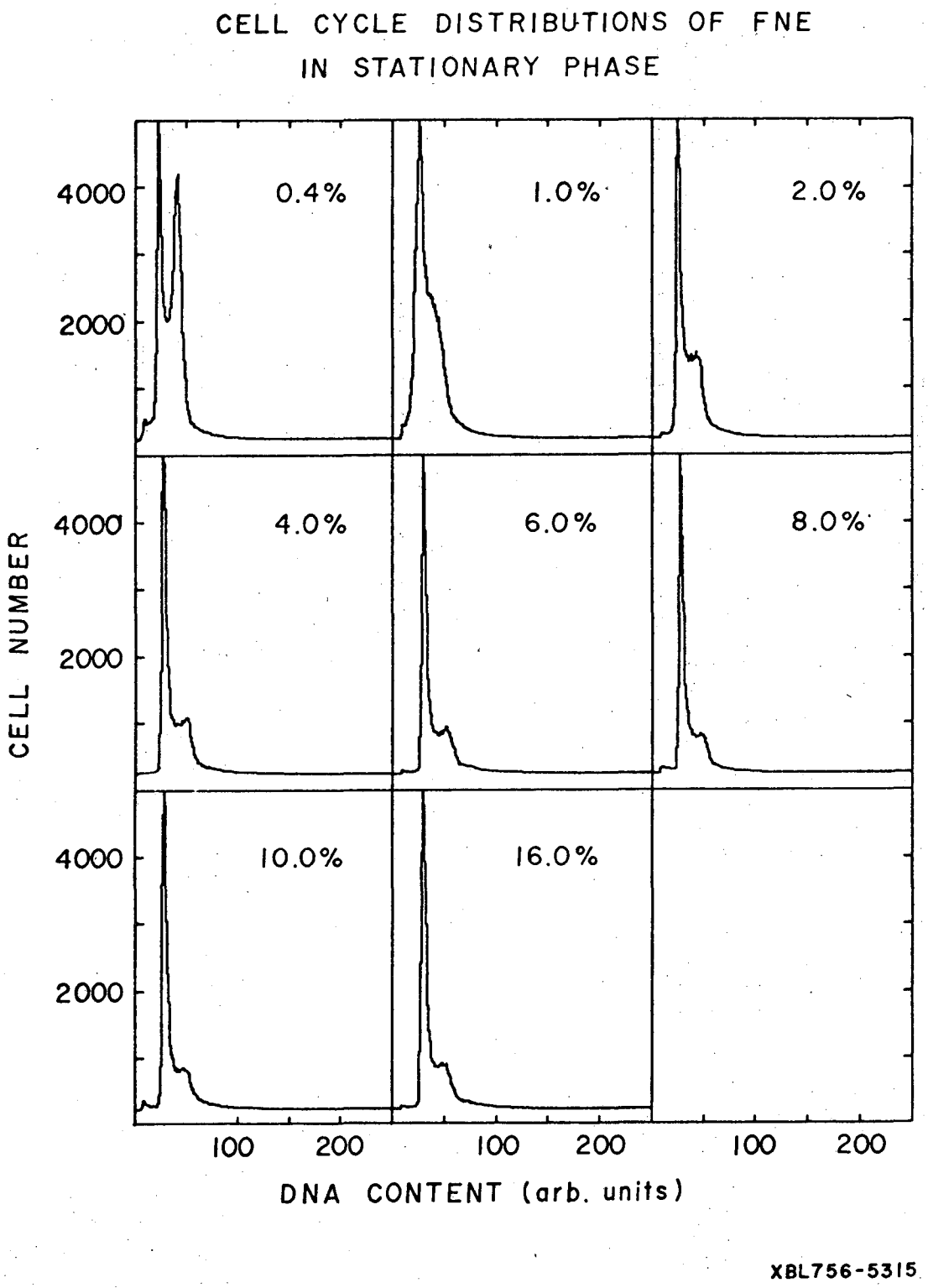

Fig. 5

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