8/15/80

UC-48

LBL-10836 c. 1
Preprint

Lawrence Berkeley Laboratory
UNIVERSITY OF CALIFORNIA

CHEMICAL BIODYNAMICS DIVISION

Submitted to the Journal of Supramolecular Structure

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April 1980

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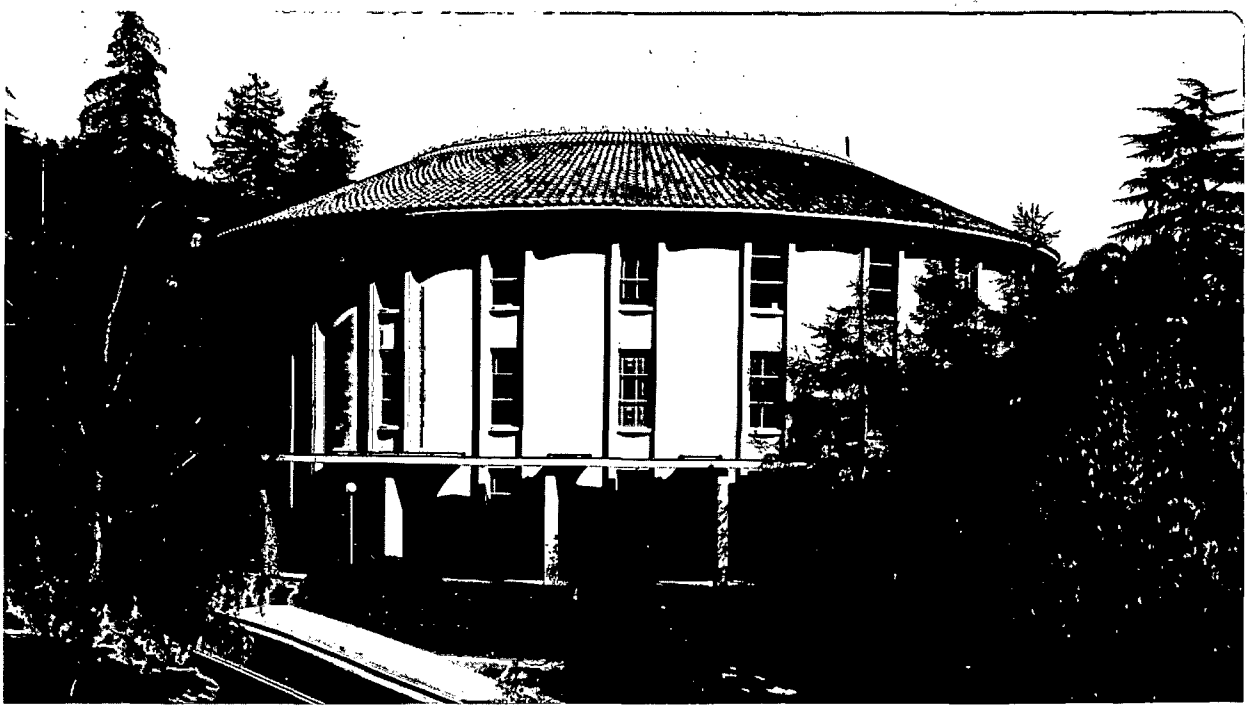

Prepared for the U.S. Department of Energy under Contract W-7405-ENG-48

LBL-10836 c. 1

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ALTERATIONS IN DNA SYNTHESIS IN MOUSE LIVER CELLS CAUSED BY THE TUMOR PROMOTER 12-O-TETRADECANOYLPHORBOL-13-ACETATE(TPA).

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Running Title: TPA Effects on DNA Synthesis

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Abbreviations: 12-O-Tetradecanoylphorbol-13-Acetate, TPA; 3H-Thymidine, 3H-Tdr; Flow Cytometry, FCM.

Abstract

We have been studying the effects of TPA on the movement of cells around the cell cycle. We monitored cell cycle position by flow cytometry as well as 3H-thymidine incorporation into DNA. When TPA was added to the culture medium(5×10^{-6}M) of growing cells, the first observed change in cell cycle distribution was a block in the movement of cells out of G_1 into the beginning of S. This effect was seen by 2 hours after adding the compound to the medium. This early block in movement out of G_1 eventually resulted in a depletion of cells in S. The depleted S was refilled with cells moving from G_1 beginning at 10 hours after TPA addition. This renewed movement of cells through S was partially synchronous. When quiescent cells were stimulated by serum in the presence of TPA, the kinetics of cell cycle movement depended on when relative to serum stimulation the TPA was added. When TPA was added at the time of serum stimulation the wave of cells moving from G_1 to S was more synchronous than controls. There was no evidence that more cells moved through the cycle in the presence of TPA than in its absence. Addition of TPA just as the cells began entering S again caused the cells in G_1 to delay their entrance into S for approximately 10 hours. These studies indicate that TPA causes an increase in synchrony of cycling cells by creating a temporary pause to cell cycling in G_1.

INTRODUCTION

Carcinogenesis is a multistep process(1-3). In the case of chemical carcinogenesis at least two steps have been identified. The first step, initiation, has received considerable attention and has been shown to involve chemicals that bind to DNA(4-6). These compounds inhibit DNA synthesis with an effectiveness that correlates with their carcinogenicity(7). The second step in chemical carcinogenesis that has been identified is promotion. Promotion is caused by chemicals which by themselves are not carcinogenic, but when added to cells at the time of, or after, initiation, greatly enhance tumorigenesis(8-11). Promotion was first characterized using an in vivo mouse epidermis assay(8,10) and has since been observed in other tissues such as liver(12), bladder(13), and colon(14). Recently, promotion has also been demonstrated in the C3H 10T 1/2 cell culture transformation system(15, 16).

The types of compounds that cause promotion are extremely heterogeneous. Many promoters are growth factors(9) which suggests that the mechanism of promotion involves some aspect of the growth regulatory machinery. Other promoters are natural compounds extractible from various plant sources. The most potent promoters known are the diesters of the tetracyclic diterpene phorbol. In this group, the most active promoter identified is 12-O-tetradecanoyl-13-acetate (TPA)(17).

The effects of TPA on various aspects of cellular biochemistry have been examined(18-20). Prominent among the effects observed is the stimulation of functions which normally correlate with preparation for the onset of DNA synthesis(21-26). Many of the studies with promoters have concerned their effect on DNA synthesis(9). However, the effects of TPA on DNA synthesis are complex. In general, the observations from many different cell systems are that TPA causes a temporary inhibition of DNA synthesis followed by a recovery to either control levels or higher(27-31). Perturbations of DNA synthesis in populations of cells *in vivo* or *in culture* are complex phenomena that can only be described completely after detailed cell cycle kinetic studies. This report describes such a study using flow cytometry(FCM) to analyze the effects of TPA on DNA synthesis in mouse liver cells(NMuLi cl 8).

MATERIALS AND METHODS

Cells and Culture Techniques

The cells used in this study were derived from MuLi(32) mouse liver epithelial cells by the cloning technique of Puck, et al. (33). All cells were cultured in plastic dishes(Falcon, Oxnard, Calif.) and incubated at 37° in a 5% CO_2 incubator. The medium used to grow the cells was Eagle's minimal medium(34; GIBCO, Grand Island, N. Y.) containing 10% donor calf serum(Flow Laboratories, Rockville, MD) and 10 ug/ml insulin(Schwarz/Mann, Orangeburg, N. Y.). The cells were judged free of mycoplasma by incorporation of 3H-thymidine (3H-Tdr 20.1 Ci/mM; New England Nuclear, Boston, Mass.) into the nucleus of cells and not the cytoplasm. Stock culture were maintained by subculturing the cells twice weekly at a cell density of 1×10^4 per cm^2. Cell counts were determined by using a Model ZBI Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

The cells were synchronized in G_1 by allowing them to grow to saturation density. Cells were seeded at 5×10^5 per 100 mm dish in medium containing 10% serum, and allowed to grow for 4 days. At saturation density, they were distributed in the cell cycle with 69% in G_1, 14% in S, and 17% in G_2+M. To stimulate the cells they were reseeded in fresh dishes with fresh medium containing 20% serum. The TPA was added at the times indicated in the experiment. TPA was dissolved in methanol and stored at -20° as a 1.0 mg/ml

solution. Methanol was added to the control cultures at the same concentration as in the experimentals(0.1%).

Flow Cytometry

The technique used for monitoring cell cycle position was flow cytometry (FCM). FCM has been described in detail elsewhere(35-37) and in these experiments a fluorescent DNA probe was used to identify where individual cells were located in the cell cycle. FCM has many advantages over 3H-thymidine incorporation for measuring cell cycle parameters(38, 39), but most importantly for these studies, FCM measures the synchrony of a population of cells moving through the S phase.

Cells were stained with propidium iodide using the technique described by Crissman and Steinkamp(40). The DNA content of the stained cells was analyzed using a flow cytometer as described previously(35). Analysis of the resulting histograms was carried out using a program developed by Pearlman, et al. (41), which is based on the approach described by Fried, et al. (42) and allows for interactive processing of data after transforming to log space, allowing for variation in spacing between G_1 and G_2+M, and extraction of a representative G_1 spread function from samples having a low contribution due to cells in S. In test cases with or without TPA, this data analysis technique was within experimental error of standard autoradiography techniques for estimating the proportion of a

population in the S phase of the cell cycle.

RESULTS

Effects on Growing Cells

NMuLi cl 8 cells have a normal doubling time of 15.0 hrs and an average residence time in G_1 of 3.9 hrs, S of 7.6 hrs, and G_2+M of 3.5 hrs(43). Figure 1 shows that the growth of NMuLi cl 8 cells in medium containing 10% serum was not affected by TPA at 5×10^{-6}M. The doubling time and saturation density of cultures is the same with or without TPA regardless of the serum concentration; however, at low serum concentrations the saturation density was slightly reduced. Flow cytometric analyses of the cell cycle distribution of cells treated with this concentration TPA show pronounced perturbations in cell kinetics shortly after TPA addition(Fig. 2). The TPA induced cell cycle redistributions are only temporary and occurred within the first doubling time of the population. The effects seen with the first addition of TPA were not prolonged when a second TPA addition was made either 4 or 24 hrs after the first addition. This observation suggests that the cells become refractory to TPA after the first addition. Reducing the concentration of TPA caused a proportional variation in the magnitude of the cell cycle redistributions, but did not alter the type of perturbations seen(data not presented).

The types of cell cycle redistributions caused by TPA addition to actively growing NMuLi cl 8 cells were quantified as described above. Initially there was an increase in

the proportion of the population in G_2+M with a concomitant decrease in the fraction of the population in G_1(Fig. 3). By 4 hr after adding TPA there was a pronounced decrease in the fraction of the population in the beginning of S. This depletion of S was paralleled by an increase in G_1 until about 8 hr when a wave of cells began to move from G_1 through S. This wave of cells was more synchronous than the untreated population. All of these perturbations were gone by 24 hr after TPA treatment.

The cell cycle redistributions described in Figure 2 could result from any one or a combination of the following TPA induced cell cycle kinetic affects. TPA could induce a stimulation of the rate of transit through S, a temporary G_2+M block, or a temporary G_1 block. To investigate the effect of TPA on the rate of DNA synthesis, cells were pulsed for 15 min with 3H-Tdr at the time when the TPA effect was expected to be maximal. The amount of incorporation of 3H-Tdr per 10^6 S phase cells was determined as a measure of the rate of DNA synthesis. The data in Table 1 show that TPA did not stimulate DNA synthesis. In fact, there was a slightly lower rate of DNA synthesis in the presence of TPA when compared to the control.

TPA inhibition of the exit of cells from G_2+M is suggested by the early increase in the proportion of cells in that phase of the cell cycle and the simultaneous decrease in the G_1 fraction. The inhibition of exit from G_2+M, how-

ever, is not sufficient to explain the decrease in the proportion of the population in the beginning of S. The decrease in S began at or earlier than 2 hr after adding TPA. As seen in Figure 2, the 2 hr point already shows a reduction in the beginning of S when compared to the 0 hr point. These effects on S were too early to be accounted for by the block in G_2+M. The earliest a G_2+M block could result in effects on S is the length of G_1, which for these cells is 3.9 hrs(43). Also, the possibility exists that the increased proportion of the population analyzed as G_2+M cells was actually due to a TPA induced clumping of G_1 cells as has been observed in other systems(Bartholomew, Farson, and Bissell, unpublished observation). The reduction of the fraction of cells in S must be due, in part, to a reduction in the transit through G_1.

TPA Effects on Serum Stimulated Cells

NMuLi cl 8 cells at their saturation density are distributed primarily in G_1(43). Reseeding in fresh medium with fresh serum results in the stimulation of cells out of G_1 into S with a lag characteristic of quiescent cells (44). This system was used to study the effect of TPA on the progress of cells through G_1. When TPA was added at the time of serum stimulation, cells entered S with about the same 12 hr lag as seen in the controls(Fig. 4). The movement through S of the TPA treated cells, however, was more synchronous than the control cells. By 14 hrs after serum

stimulation and TPA addition most of the stimulated cells were moving as a tight cohort through the end of S. In the controls, the 14 hr distribution was considerably more spread out in S. Colchicine(0.02 ug/ml) addition at 10 hr after serum addition trapped the stimulated cells in mitosis. The kinetics of buildup behind the colchicine block revealed that TPA did not alter the number of cells stimulated by serum(data not presented). When TPA was added 12 hr after serum stimulation the effect was analogous to that seen when TPA was added to randomly growing populations(Fig. 2). About 4 hr after addition of the compound the entrance of cells into S from G_1 was reduced. Cells that were in S or in late G_1 at the time of TPA addition continued through S and into G_2+M normally.

To determine whether TPA was acting throughout the G_o to S period, the addition of TPA to serum stimulated cells was delayed for different time periods. Figure 5 shows the effects on cell cycle kinetics of adding TPA at 0, 2, and 4 hrs after serum stimulation. When TPA was added at times shortly after serum stimulation, the entrance of cells into S was delayed relative either to control cells or cells treated with TPA at 0 hr. The effect was to increase the synchrony of the population as the TPA was added at later times after serum stimulation. If the TPA addition was delayed until 8 hrs after the serum stimulation, the effect was to hold back some of the cells from entering S. Some cells did progress into S with normal serum stimulation

kinetics, but others were delayed approximately 4 hrs in their entrance into S(data not presented). These results suggest that the TPA sensitive part of G_1 is not identical to the serum dependent G_0 to G_1 transition(45), and that serum stimulated cells are still sensitive to TPA inhibition of G_1 transit until about 4 hr before the beginning of S.

DISCUSSION

The TPA induced cell cycle redistributions reported here are consistent with the model presented in Figure 6. This model states that TPA induces a temporary halt to progression through G_1 for both actively growing cells or for cells stimulated by serum. The TPA induced block(G_o') is not the same as the block to G_1 transit seen with medium limitation(G_o) because TPA is maximally active if added after serum stimulation has begun. There is a point approximately 4 hrs before the beginning of S when cells become refractory to the TPA induced cell cycle delay. This point probably corresponds to the beginning of G_1 in actively growing populations, and may separate G_o related "lag" events from G_1 events.

Whether G_o' is a normal step in the progress of cells from G_o to S is not indicated by these experiments. TPA does not appear to act on the commitment step normally stimulated by serum since it does not affect the proportion of the population stimulated by serum to enter S.

Modeling of the cell cycle effects of TPA are made especially difficult by the observation that the cells are becoming refractory to the TPA. The results of the readdition experiments suggest that TPA is not being metabolized; however, the first addition of TPA could induce a highly active metabolic system which quickly destroys the second batch of TPA. Other physiological effects of TPA have also

been shown to become refractory to the TPA effects(46, 47).

Temporary inhibition of DNA synthesis by TPA has been demonstrated in several other cell culture systems(27-31). In most of these systems the inhibition is followed by a stimulation of DNA synthesis as measured by 3H-thymidine incorporation. The results reported here indicate that TPA does not have a direct effect on DNA synthesis, but instead temorarily inhibits G_1 cells from entering S. This temporary inhibition results in a synchronization of G_1 cells which when they move into S increase the amount of DNA synthesis occurring in the culture relative to the controls. The block to G_1 transit is not at the level of the G_o to G_1 commitment(44) since TPA does not alter the proportion of the population moving through the cell cycle.

The relationship of these observations to tumor promotion is not known. It is not likely that promoters are altering the ability of carcinogens to initiate transformation since Poiley et al. (48) have demonstrated that TPA added to hamster embryo cells at the same time as or before 3-methylcholanthrene inhibits the transformation efficiency. Promotion is more likely to result from the alteration of events that occur after initiation. Possibly promoters activate the so-called "persistent lesions" in DNA(49), or stimulate the expression of repressed information as has been recently demonstrated by Soprano and Baserga(50) for mouse-human hybrids. The involvement of cell cycle syn-

chronization in these events is being studied.

ACKNOWLEDGEMENTS

We thank Jean Lawson and Maria Costin for technical assistance, Catherine Gisser for analyzing the FCM samples, and Alicia Sarazoza-Tainer for help with the illustrations. A special thanks goes to Hisao Yokota for invaluble discussions throughout the course of this work. This research was carried out under the auspices of the Division of Biomedical and Environmental Research, United States Department of Energy Contract W-7405-ENG-48.

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TABLE 1. EFFECTS OF TPA ON DNA SYNTHETIC RATES

SAMPLE	PMOLES TDR/15MIN/10^6 S CELLS
CONTROL	131
TPA	99

ACTIVELY GROWING CULTURES OF NMULI CL 8 CELLS WERE TREATED WITH 5×10^{-6} M TPA AT 0 TIME. AFTER 2 HRS THE CULTURES WERE PULSED FOR 15 MIN WITH ^{3}H-TDR (20 CI/MMOLE). AT THE END OF THE PULSE THE CELLS WERE HARVESTED AND FIXED. ALIQUOTS WERE TAKEN FOR FCM ANALYSIS, CELL COUNTS, AND DETERMINATION OF INCORPORATED ^{3}H-TDR. THE PROPORTION OF THE POPULATION IN S WAS DETERMINED FROM THE DNA HISTOGRAM.

FIGURE LEGENDS

Figure 1. Growth of NMuLi cl 8 in Medium Containing TPA.

NMuLi cl 8 was seeded in medium containing either 10% serum( ) or 0.5% serum( ). After 24 hrs TPA(5 x 10^{-6}M) was added to half the cultures at each serum concentration. Methanol was added to the remaining cultures(0.1% final concentration) as a solvent control. Cells were harvested by trypsinization and counted. The TPA containing cultures are represented by shaded symbols.

Figure 2. TPA Induced Cell Cycle Redistributions of Actively Growing Cells.

TPA(5 x10^{-6}M) was added at time 0 and cells were harvested and analyzed as described in MATERIALS AND METHODS.

Figure 3. Quantification of TPA Induced Cell Cycle Redistributions.

The data from Figure 2 was analyzed as described in MATERIALS AND METHODS.

Figure 4. Addition of TPA to Serum Stimulated Cells.

NMuLi cl 8 cells were grown to their saturation density in medium containing 10% serum. The cells were stimulated by transferring into fresh medium and 20% serum. The TPA concentration was 5 x 10^{-6}M.

Figure 5. TPA Added at Various Times After Serum Stimulation.

TPA(5 x 10^{-6}M) was added at the times indicated.

Figure 6. Model for TPA Effects During G_1.

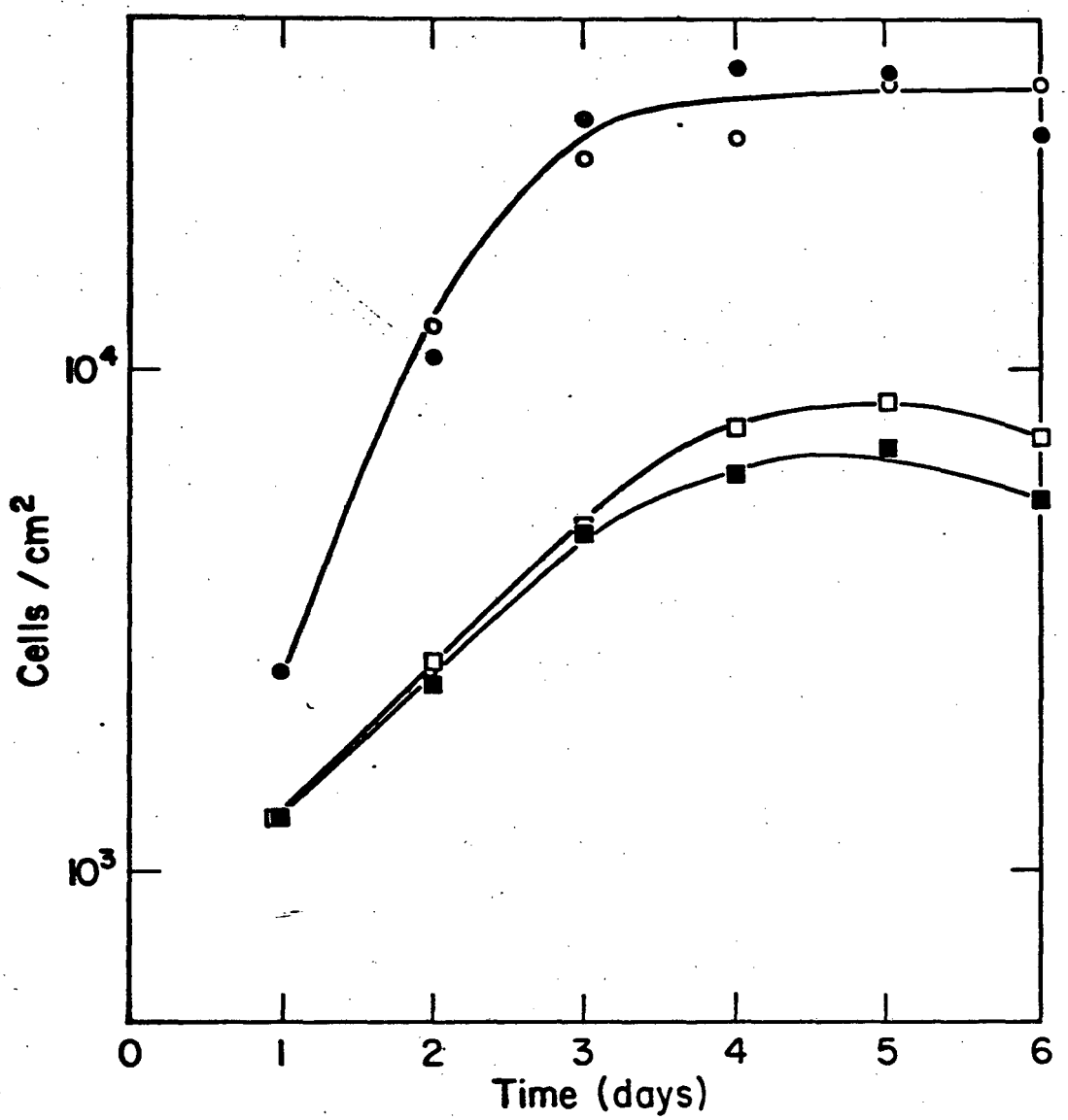

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Figure 1.
Bartholomew, et al.

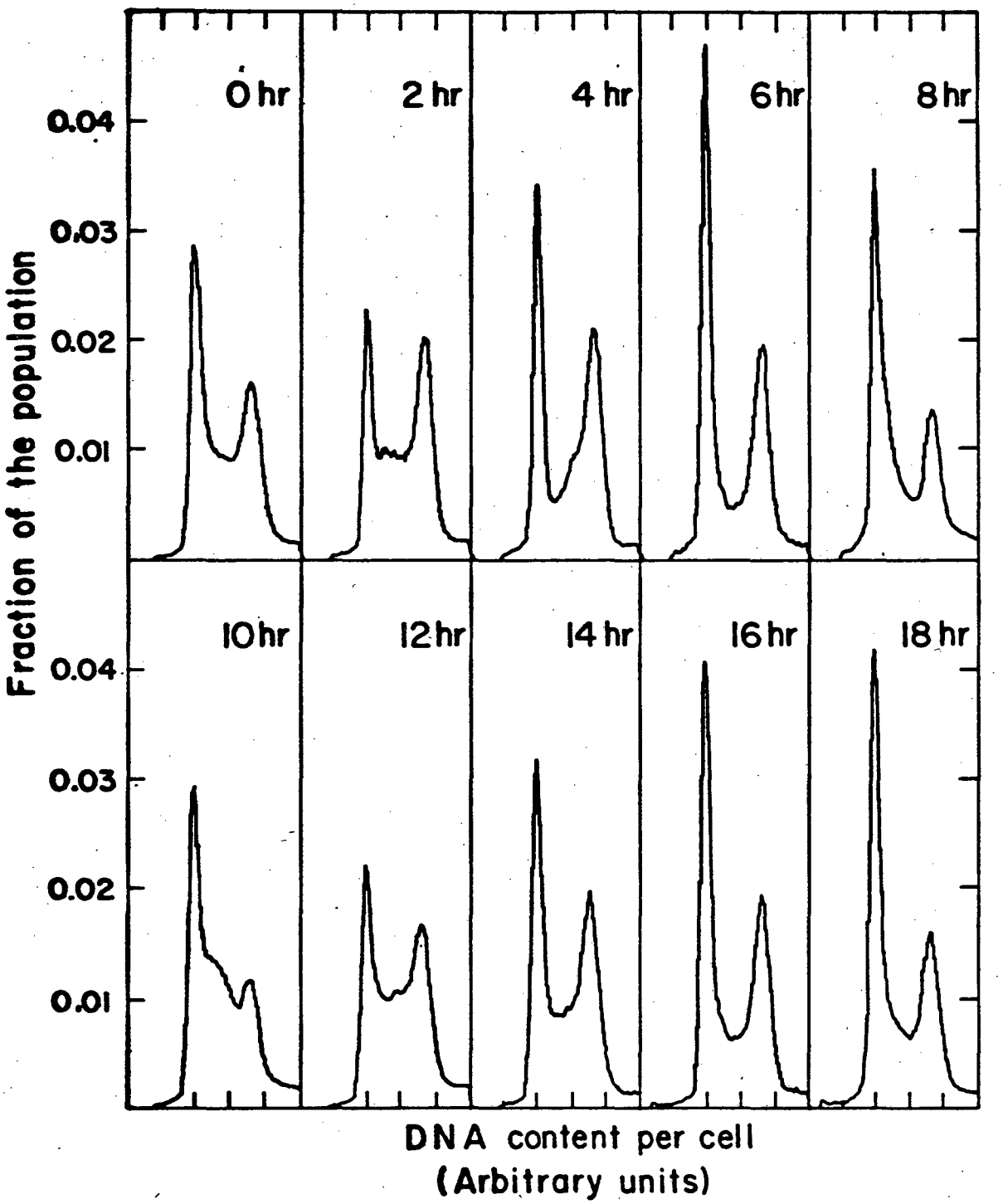

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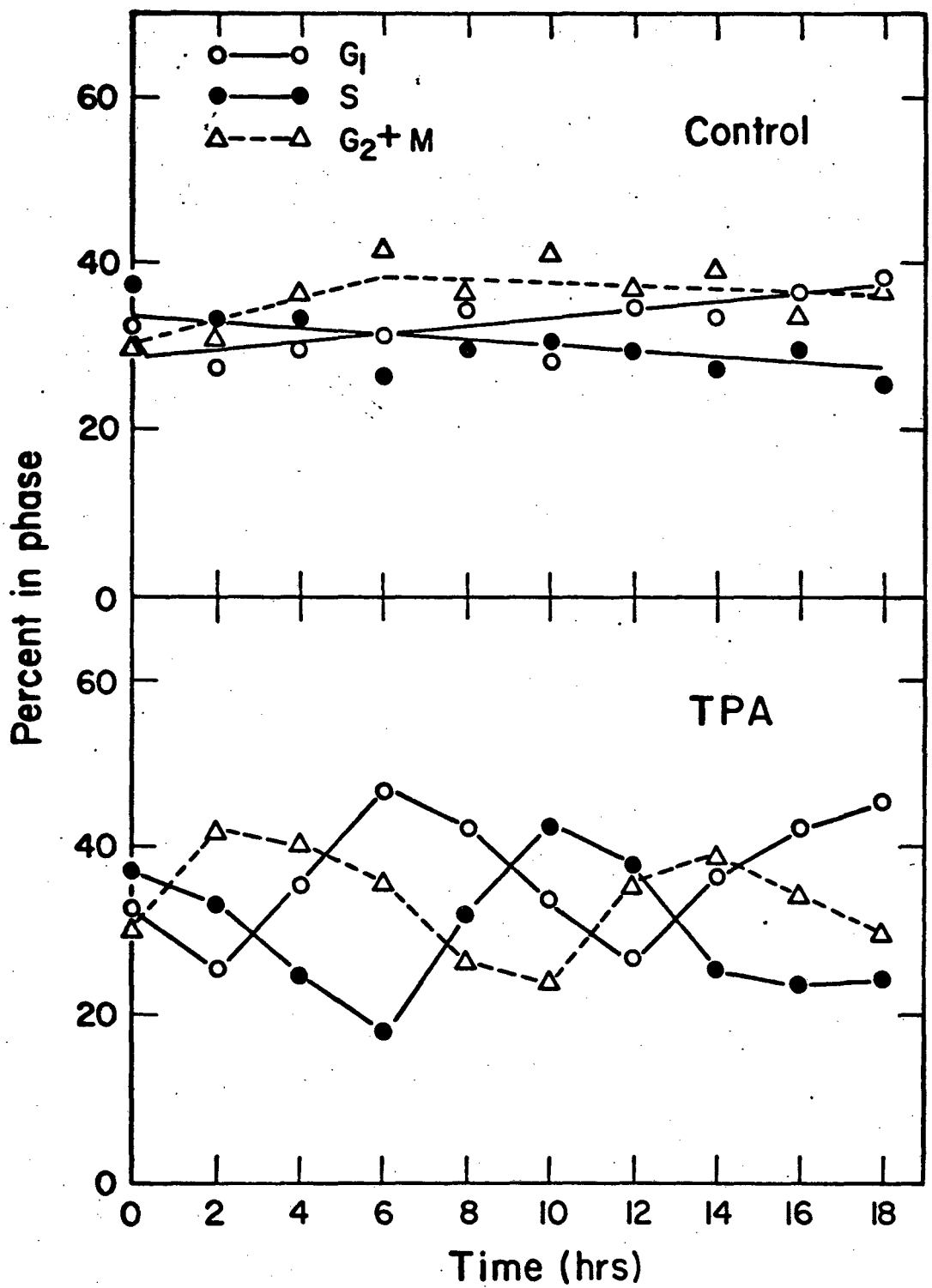
Figure 2.
Bartholomew, et al.



XBL 802-4053

Figure 3.
Bartholomew, et al.

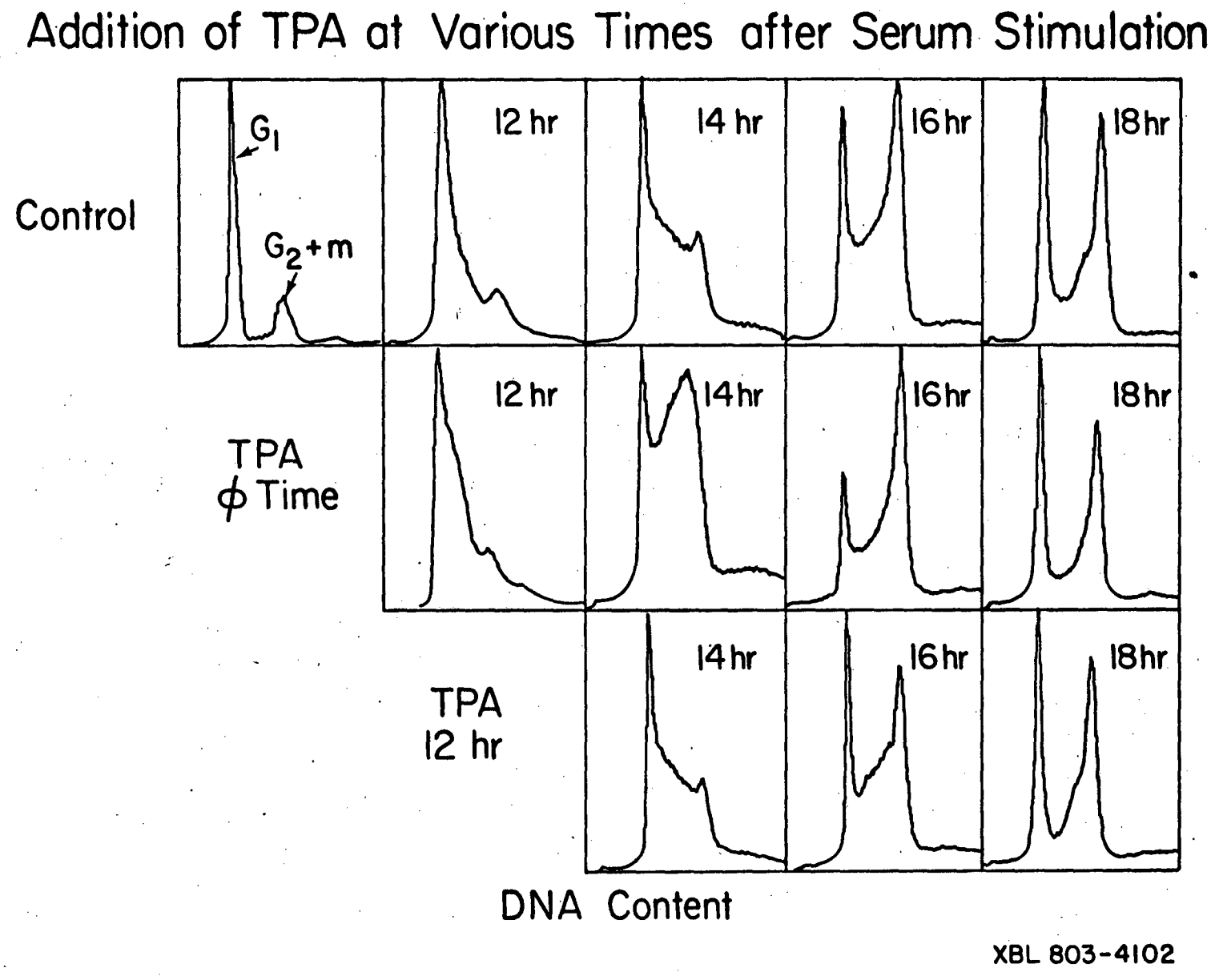


Figure 4.
Bartholomew, et al.

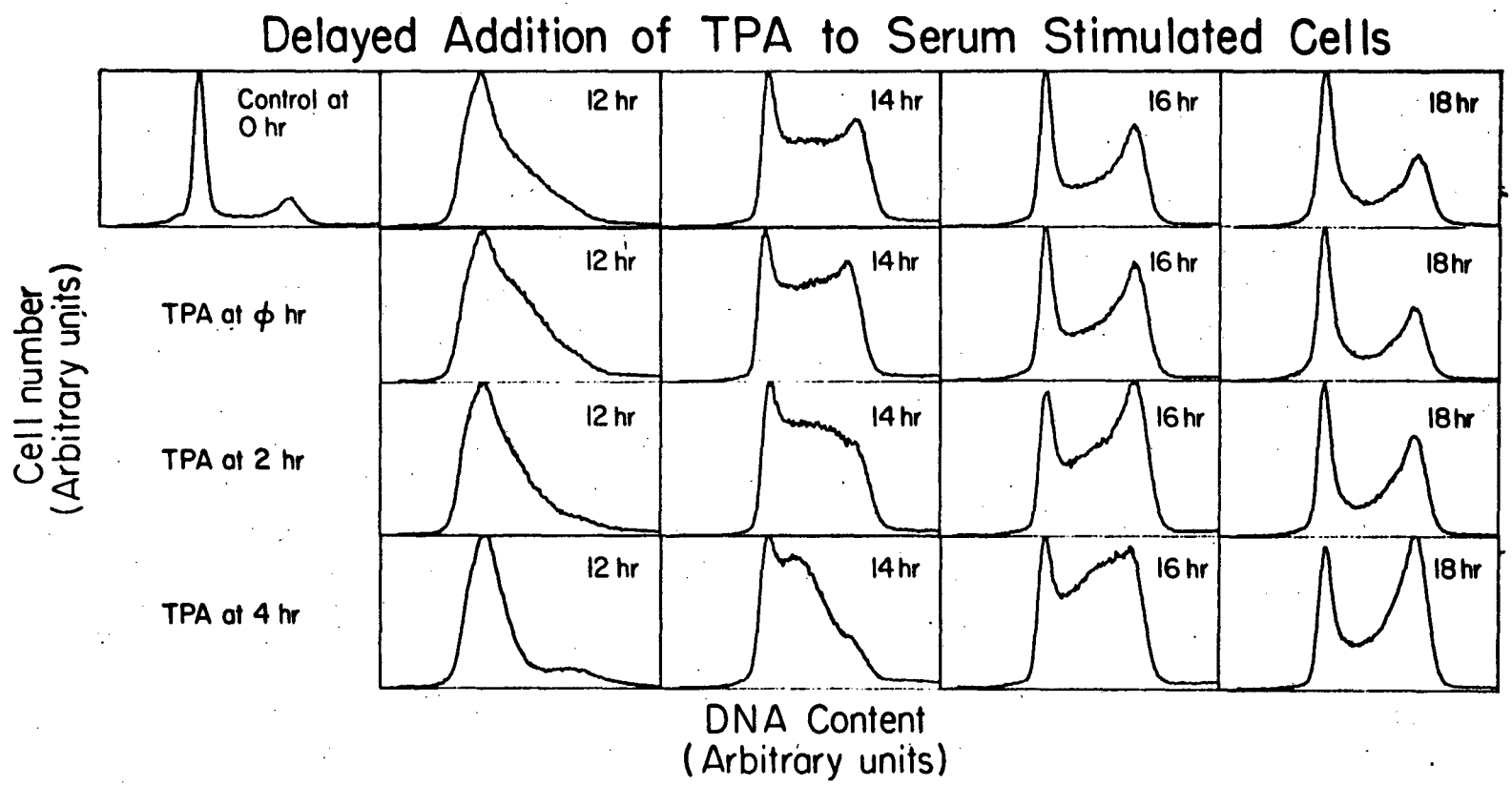


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Figure 5.
Bartholomew, et al.

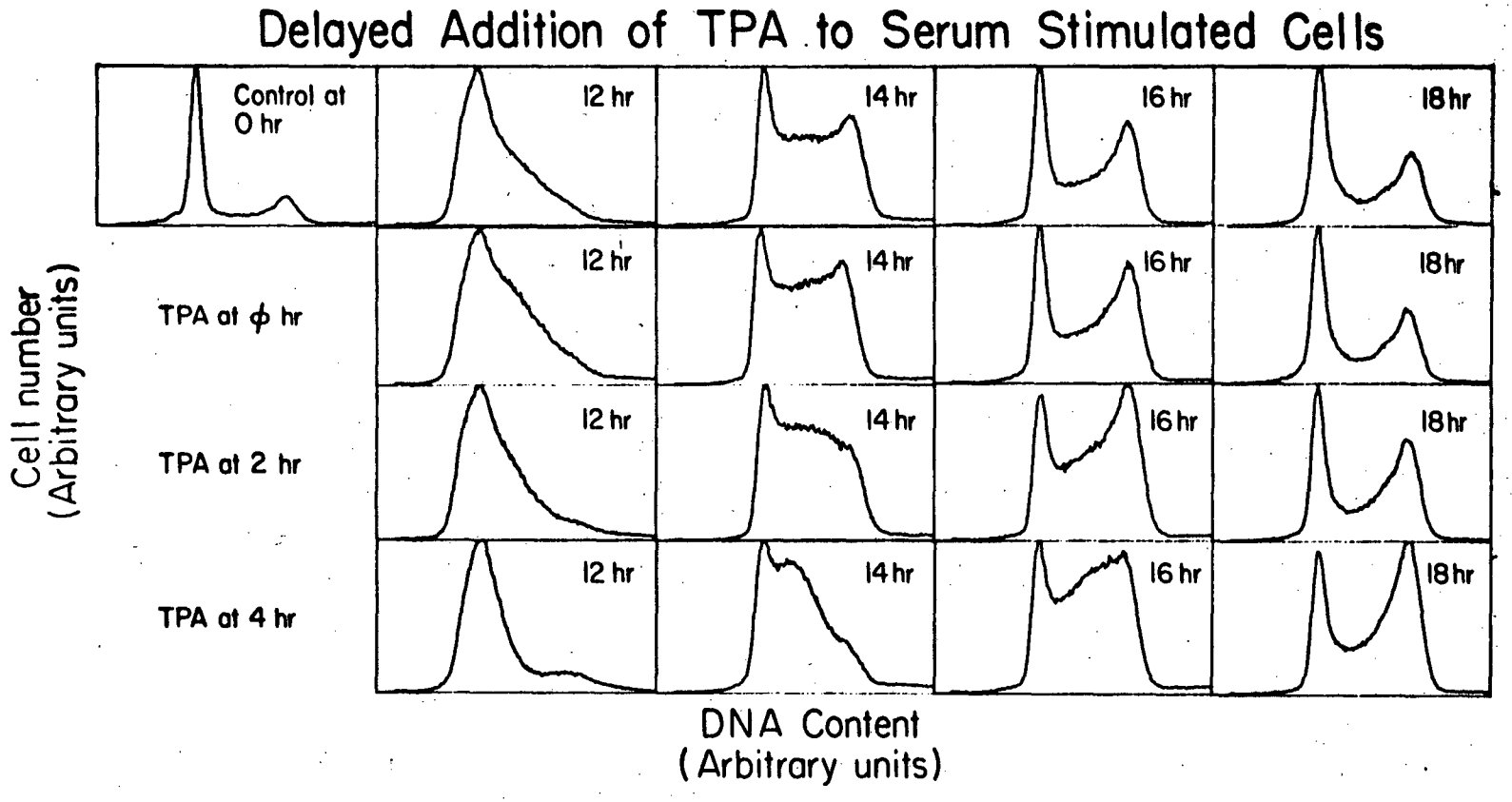

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Figure 5.
Bartholomew, et al.

Model for TPA Effects During G_1

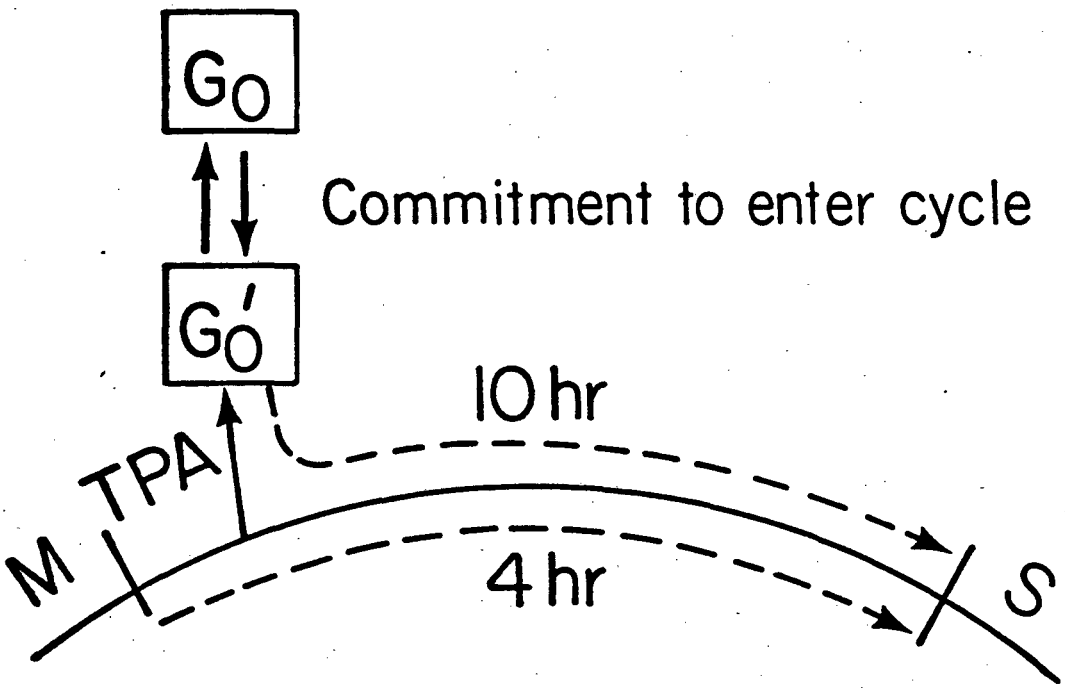

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Figure 6.
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