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INITIATION AND COMPLETION OF MITOSIS IN HELA CELLS IN THE ABSENCE OF PROTEIN SYNTHESIS

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HeLa cells were found to enter and complete mitosis in the presence of puromycin or actinomycin D. Daughter cells formed in the presence of puromycin contained completed nuclear membranes. Daughter cells resumed protein synthesis shortly after completing mitosis; however, this resumption of protein synthesis could be prevented by actinomycin D. Data presented here indicate that all cellular constituents necessary for successful initiation and completion of mitosis are produced prior to the onset of mitosis. They also indicate that new messenger RNA synthesis is required for resumption of protein synthesis after mitosis, and that the inhibition of protein synthesis observed in mitotic mammalian cells is a result of messenger RNA depletion.

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The inhibition of protein and RNA synthesis during mitosis is now a well documented phenomenon (Johnson and Holland, 1965; Marcus and Robbins, 1963; Monea, 1964; Prescott and Bender, 1962; Taylor, 1960; Terasima and Tolmack, 1963). The marked decline in protein synthesis appears to be due to the unavailability or non-functioning of host cell messenger RNA, since mitotic cells are able to produce virus (Johnson and Holland, 1965), and polysomes can no longer be found in their cytoplasm (Scharf and Robbins, 1966). The cell must, therefore, form and dissociate a spindle apparatus, move and disaggregate chromosomes, and separate into two daughter cells in the absence of any detectable RNA or protein synthesis. Such a task requires that either some messenger RNA remain during mitosis to code for any enzymes which may be necessary for the completion of cell division, or that the cell be completely pre-programmed for mitosis. Evidence to be presented here indicates that HeLa cells are able to initiate and complete mitosis in the absence of RNA or protein synthesis. Evidence is also presented that new RNA synthesis is required in order for the newly formed daughter cells to resume protein synthesis at normal levels.

Monolayers of HeLa cells were synchronized by growth for 12 hours in Eagle's medium containing 2 mM thymidine (Xeros, 1962). The monolayers were then washed with 37°C Hank's balanced salt solution (BSS) and incubated for 10 to 13 hours in fresh 37°C Eagle's medium without thymidine. The monolayers were examined periodically and the mitotic cells harvested at the peak mitotic index by vigorous shaking in 37°C BSS. More than 90% of the cells collected in this manner were in metaphase or later stages of mitosis.

Table 1 shows that cells growing in Eagle's medium will enter and complete mitosis in the presence of puromycin and actinomycin D. In fact, pre-treatment of synchronized monolayers with either actinomycin D or puromycin for 70 min prior to harvesting did not prevent the cells from entering and completing mitosis. Cells were observed going through mitosis after treatment of the monolayers with either of these inhibitors for up to two hours before the onset of mitosis. Harvesting mitotic cells after such prolonged treatment with inhibitors was not possible as the treatment loosened the entire monolayers. Note also that cells were able to complete mitosis at 4°C.

The above results might arise if mitotic cells were impermeable to puromycin. To test this possibility, cells were arrested and collected in metaphase using 0.05 µg/ml of vinblastin sul-

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Table 1
Entry into, and completion of mitosis by HeLa cells incubated in the presence of puromycin, actinomycin D or at low temperatures.

Treatment	Cell no. at time of harvesting $\times 10^{-4}$	Cell no. 60 min after harvesting $\times 10^{-4}$
None	120	210
Puromycin 100 $\mu\text{g}/\text{ml}$ after harvest	120	200
Actinomycin D 50 $\mu\text{g}/\text{ml}$ after harvest	120	205
Puromycin 20 $\mu\text{g}/\text{ml}$ 70 min pre-harvest, 100 $\mu\text{g}/\text{ml}$ post harvest	192	382
Actinomycin D 5 $\mu\text{g}/\text{ml}$ 70 min pre-harvest, 50 $\mu\text{g}/\text{ml}$ post-harvest	108	172
Incubated at 40°C immediately after harvest*	65	120

Interphase cells were thymidine-synchronized, and mitotic cells collected as described in the text. Actinomycin D or puromycin was added to the cells at the designated time before, or immediately after harvesting. Total cell counts were made at the time of harvesting and 60 min later.

* Cells incubated at 40°C were harvested and placed in a test tube and immediately immersed in a 40°C water bath so that they reached 40°C within 20-30 seconds.

fate, then infected with poliovirus at a multiplicity of 10 plaque forming units (PFU)/cell according to the procedure of Johnson and Holland (1965). A total of 1.1×10^6 infected metaphase arrest cells were placed in plaque bottles. Puromycin (20 $\mu\text{g}/\text{ml}$) was added to the appropriate bottles, and the cells incubated for 24 hours. Vinblastin sulfate was present at all times. Table 2 shows that puromycin was able to act on metaphase-arrest cells and prevent virus production. Therefore, mitotic cells do not appear

to be refractile to the action of puromycin.

Fig. 1 shows electronmicrographs of mitotic cells treated as in table 1. The mitotic cells were harvested and centrifuged. Most of these mitotic cells were in metaphase. One sample of cells was immediately fixed in 2% osmium tetroxide as a zero time control (fig. 1a). The remainder of the cells were divided in half, one half was incubated in Eagle's medium, and the other half was incubated in medium containing 20 $\mu\text{g}/\text{ml}$ of puromycin. After one hour, both samples were fixed and stained with lead citrate and uranyl acetate. No differences were noted in the cells completing mitosis in the presence or absence of puromycin. Fig. 1b clearly demonstrates daughter cells separating in the presence of puromycin. Note the characteristic nuclear membrane forming around the daughter cell chromatin.

If significant amounts of functional messenger RNA are maintained throughout mitosis, then resumption of protein synthesis in daughter cells leaving mitosis might be independent of new RNA synthesis. Conversely, if the resumption of protein synthesis is programmed primarily by newly-synthesized messenger RNA, then actinomycin D should prevent the renewal of protein synthesis in daughter cells. These possibilities were examined in the following experiment: Mitotic cells were harvested, resuspended in conditioned Eagle's medium, and distributed among two sets of tubes, each tube receiving 10^6 cells. One set was treated with actinomycin D, 5 $\mu\text{g}/\text{ml}$, and the second set was untreated. A third set of tubes containing 10^6 metaphase arrested cells/tube in conditioned media was prepared by treating monolayers with vinblastin sulfate, 0.05 $\mu\text{g}/\text{ml}$, and harvesting the mitotic cells as described previously. At designated times after harvesting, one tube from each set was pulse-labeled for 20 minutes with 0.5 $\mu\text{C}/\text{ml}$ of C^{14} phenylalanine (393 mC/mM). The indi-

Table 2
Effect of puromycin on poliovirus production by HeLa cells arrested in mitosis.

Treatment	Number of cells per tube	Total plaque forming units of virus produced	Plaque forming units per cell
0.05 $\mu\text{g}/\text{ml}$ vinblastin sulfate only	1.1×10^6	150×10^7	1300
0.05 $\mu\text{g}/\text{ml}$ vinblastin sulfate plus 20 $\mu\text{g}/\text{ml}$ puromycin	1.1×10^6	15×10^5	1.3

Cells were arrested in mitosis, harvested, and infected as described in the text. Puromycin, 20 $\mu\text{g}/\text{ml}$, was added to the appropriate cells and vinblastin sulfate, 0.05 $\mu\text{g}/\text{ml}$ was present in every tube at all times. Virus was harvested and titered 24 hours after infection.

Fig. 1. Daughter cells formed in the presence of puromycin. HeLa cells were harvested as explained in the text and either (a) fixed immediately, or (b) incubated for 60 min in Eagle's medium plus 20 $\mu\text{g/ml}$ puromycin. Note in (1b) that daughter cells have nearly separated and nuclear membranes have been formed in both daughter cells (arrow). Magnification: 10 200 $\times$.

Fig. 2. Resumption of protein synthesis in cells leaving mitosis in the presence and absence of actinomycin D. Cells were treated and harvested as tubes at a concentration of 10^6 /ml. Actinomycin D was in the appropriate tubes at a concentration of $6 \mu\text{g}/\text{ml}$. At the stated times, after incubation, the cells were given a 20 min pulse of C^{14} phenylalanine, sp. activity 393 mCi/mM. Actinomycin D treated interphase cells were given a 20 min pulse of phenylalanine after exposure to $5 \mu\text{g}/\text{ml}$ of actinomycin D for 120 min. The curves shown here are typical samples from independent experiments. Similar results were obtained in four other experiments.

cated inhibitor treatment was continued during labeling. The results of such an experiment are shown in fig. 2. Notice that daughter cells recover significant rates of protein synthesis very quickly after mitosis. However, treatment of mitotic cells with actinomycin D prevents protein synthesis in cells leaving mitosis from ever rising above the levels of that found in cells arrested in metaphase. Note also, that actinomycin D has little effect on protein synthesis in interphase cells. Indeed, the levels of amino acid uptake in actinomycin D and vinblastin sulfate treated cells could be accounted for entirely by a small percent of contaminating interphase cells.

These data would indicate, along with those of Went (1960), that spindle tubule protein subunits and other macromolecules necessary for cell division are synthesized prior to mitosis, and that mitosis, cytokinesis, and reformation of the daughter cell nuclear membranes do not require the synthesis of new proteins. It follows then that spindle tubules, daughter cell nuclear membranes and other cellular constituents found during and immediately after mitosis may be aggregated from subunits synthesized prior to mi-

toxis, and that a stable messenger is not necessary for directing mitosis. Also, if messenger RNA synthesis declines 2 hours or more prior to prophase, then the loss of polysomes prior to mitosis may involve little more than the natural decay of messenger RNA. However, Salb and Marcus (1965) have presented evidence which indicates that an alteration of ribosomes may be a factor in the decrease in protein synthesis of mitotic cells.

Finally, it is of interest that mitotic HeLa cells complete cell division and daughter cell nuclear membrane formation in about the normal period of time, even at 40°C . This finding, taken together with earlier work on invertebrate cell energy requirements for mitosis (Mazia, 1961), would suggest that a cell entering mitosis is indeed completely preprogrammed with macromolecules and provided with available energy for the completion of a complex, intricate and vital task.

It is interesting that Gross and Fry (1966), have recently shown that sea urchin embryos which have a long-lived messenger RNA do not show a decline in protein synthesis during mitosis. This is further evidence that mitosis does not require cytoplasmic incapacity for protein synthesis.

After this work was completed, other laboratories have published work with other cell systems which also show that actinomycin, puromycin, and cyclohexamide do not block mitosis unless added hours before mitosis (Cummins et al., 1966; Tobey et al., 1966; Tobey, personal communication). The general conclusions of Tobey et al., are in agreement with our conclusions even though they used hamster cells with a different approach to cell cycle analysis, while we used HeLa cells in synchrony.

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