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FURTHER STUDIES ON A MUTANT MAMMALIAN CELL LINE DEFECTIVE IN MITOSIS

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SUMMARY

Experiments have been performed on a temperature-sensitive hamster cell line, ts-546. After the cells are switched to the non-permissive temperature, interphase cells continue through the cell cycle until the cells enter metaphase. Normal mitotic events then fail to occur. Metaphase chromosomes in the cells condense and coalesce into chromatin aggregates. Nuclear membrane re-forms around the aggregates resulting in the formation of mono-, bi- or multi-nucleate interphase-like cells. The conversion of mitotic cells to interphase-like states is completed within a few hours. The initial characterization of the mutant cell line was based on the observation that rounded-up cells accumulate in culture at the non-permissive temperature. The mitotic rounding-up process may be utilized as a useful marker for selective isolation of mutant cell lines defective in mitosis.

In a previous communication [1], the isolation of a temperature-sensitive hamster cell line was reported.

Shortly after the mutant cells were switched from the permissive temperature of 33 to 39°C, the non-permissive temperature, the cells ceased to divide. Upon further incubation at 39°C, cells were killed regardless of their density in the dishes. The reversion frequency was about 5×10^{-7}. No generalized defect in DNA, RNA or protein synthesis was found at the non-permissive temperature. At 39°C the mitotic cells were arrested in metaphase and ts-metaphase, which, under the light microscope, appeared to be similar to the c-metaphase resulting from colchicine treatment as shown by accumulation of cells with condensed metaphase chromosomes [1].

One unresolved problem was that, instead of the mitotic index increasing continuously after the cells were shifted to 39°C, the index rose for about 7 h, then decreased [1]. One possible explanation is that the mitotic cells at 39°C preferentially lyse, resulting in a decrease in mitotic index. A different explanation would be that only a small fraction of the cells is able to enter mitosis and to be transiently arrested there. The other cells would remain in interphase and round up without entering mitosis after incubation at 39°C. An alternative possibility calls for the cells to continue entering mitosis. When unable to complete mitosis at the non-permissive temperature, the cells blocked in mitosis become interphase-like, lowering the mitotic index. In this report, we report our results which provide an answer to this problem, and on the fate of the cells blocked in mitosis.

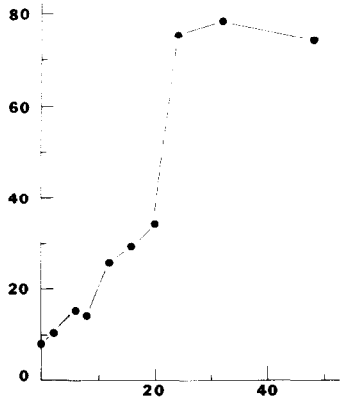

Fig. 1. Abscissa: time (hours); *ordinate:* % of cells found floating or loosely attached.
Accumulation of rounded, floating cells in ts-546 at 39°C.

MATERIALS AND METHODS

Hamster HM-1 cells and their ts-546 derivative were cultured in Dulbecco's modified Eagle's medium with 10% calf serum but without the addition of antibiotics, as described previously [1]. Cells were routinely cultured as monolayers in dishes or flasks in a controlled environmental room maintained at 33.2± 0.2°C. When a shift of temperature to 39°C was needed, the medium in the culture vessel was removed, followed by addition of medium prewarmed to 39°C. All subsequent manipulations and incubation were performed in a second controlled environmental room maintained at 39.5°C±0.2°C.

For the collection of mitotic or floating cells, flask cultures were shaken to remove cells in medium and cells loosely attached to the flask surface [2]. The number of cells removed was counted with a hemacytometer. For further microscopic examination, the cells were centrifuged and resuspended in 3 : 1 methanol–acetic acid fixative at 0°C. After the first fixation procedure, the cells were centrifuged again and resuspended in an additional amount of 3 : 1, followed by the addition of the cells to cold slides. The cells were then stained with aceto-orcein. The cell viability was determined with 0.5% trypan blue in phosphate-buffered saline. Viable cells excluded the dye while dead or lysed cells failed to do so.

RESULTS

Terasima & Tolmach found that, when a culture of monolayer cells is agitated mechanically, mitotic cells detach preferentially and can be collected as a synchronous culture [2]. The technique was utilized in this study. Mutant ts-546 cells were subcultured into flasks and grown at 33°C for 3 days when the cells were in log-phase growth. After the flasks containing the monolayer cells were shifted to 39°C, one flask each was agitated at intervals and medium containing floating cells was collected. An aliquot of cells were placed in a hemacytometer and counted. The cells remaining attached to the flask surface were trypsinized, collected, and also counted. The number of floating and loosely attached cells in flasks removed at successive intervals increased continuously (fig. 1). After 24 h, approx. 75% of the cells were loosely attached or not attached to the flask surface (fig. 1). The increase in the percent of floating cells paralleled the accumulation of cells which had changed from a fibroblast morphology to rounded in appearance.

Since mitotic cells preferentially float into medium [2], it is expected that the floating cells collected at each time interval after incubation at 39°C would be mitotic cells. When the floating cells were fixed, stained, and examined, the expectation was not confirmed (fig. 2). The floating cells removed 4 h after shifting to 39°C showed high percentage of mitotic cells. As the cells in flasks were incubated longer at 39°C before

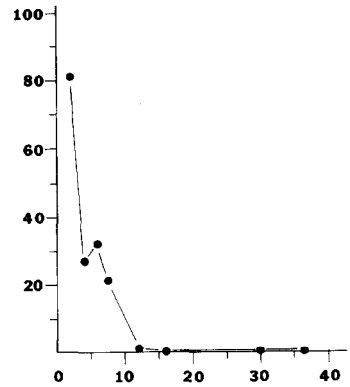

Fig. 2. Abscissa: time (hours); *ordinate:* % of cells in mitosis.
Decrease in fraction of floating cells in mitosis.

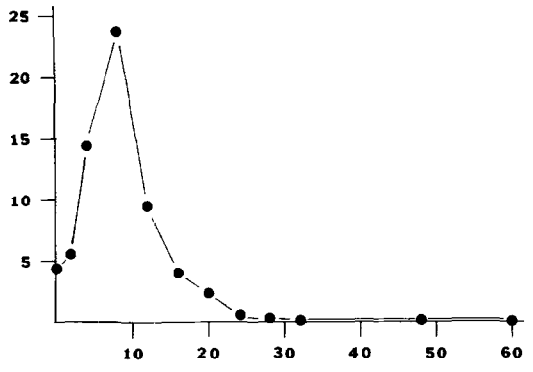

Fig. 3. Abscissa: time (hours); *ordinate:* % cells in mitosis.
Change in the % of cells in mitosis at 39°C.

the shake-off, the mitotic index of the floating cells collected decreased continuously. The index reached near zero after 24 h and remained at that level.

Similar results were obtained when cells in dishes were examined. Cells in log-phase growth at 33°C were shifted to 39°C. At intervals after the temperature shift, cells floating in medium were collected and pooled with cells removed with trypsin from the dish surface. All the cells thus harvested were spread on slides, stained, and examined. The mitotic index of the cells increased, to a maximum of 23 % at 8 h after temperature-shift. A decrease in the index then ensued (fig. 3). After the cells were at 39°C for approx. 24 h, the mitotic index was less than 1 % and remained at that level for the duration of the experiment (fig. 3).

It is conceivable that the decrease in mitotic index results from preferential lysis of mitotic cells at 39°C. Test with trypan blue showed that mitotic cells found in all experiments reported here were physiologically viable.

One possible mechanism to account for the above result is that the ts-546 cells are not blocked in mitosis at 39°C, but rather the increase in the fraction of cells floating in medium is due to interphase cells becoming defective and rounding up. An alternative possibility is that the mutant cells are blocked in mitosis, but the mitotic cells subsequently become interphase-like at the non-permissive temperature resulting in a lowering of the mitotic index. Experiments were conducted to test the possibilities.

Cells grown in a flask at 33°C were shifted to 39°C for 4 h. The flask was agitated to remove the floating and loosely attached cells accumulated during the initial 4 h. Fresh medium at 39°C was added to the flask which was then allowed to incubate at 39°C for an additional 2 h. Floating and loosely attached cells accumulated during the 2 h periods were then collected. Part of the cells so collected were fixed and stained, while the remaining cells were subcultured into dishes and further incubated at 39°C. At intervals, all the cells in each dish were harvested, fixed and stained.

Microscopic analysis revealed that the cells, which became floating or loosely attached during the 2 h period described above, contained a large percentage of

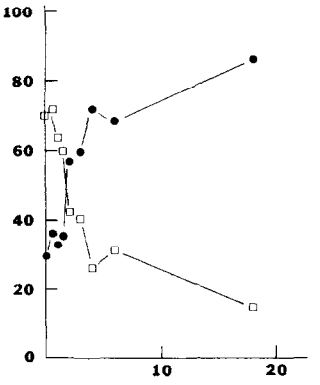

Fig. 4. Abscissa: time (hours); *ordinate:* % of cells. □---□, % of cells in mitosis; ●---●, % of interphase and interphase-like cells.
Decrease in % cells in mitosis and increase in % cells in interphase-like states after mitotic cells were collected and further incubated at 39°C.

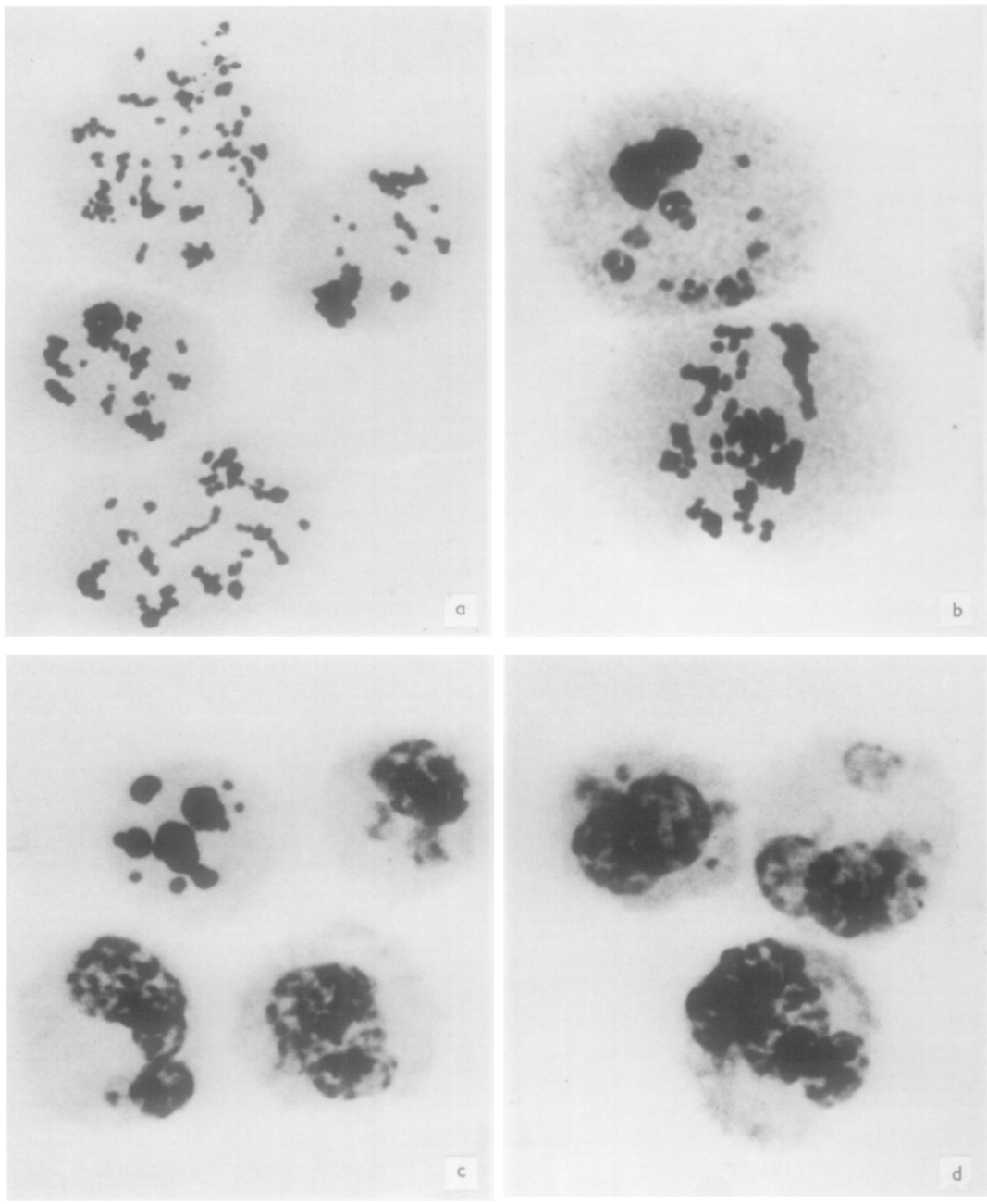

Fig. 5. Progression of mitotic cells arrested in metaphase at 39°C into interphase-like states. (*a*) ½ h; (*b*) 1½ h; (*c*) 3 h; (*d*) 4 h after the mitotic cells collected were subcultured. ×1 000.

mitotic cells containing metaphase chromosomes (zero time in fig. 4). Upon further incubation at 39°C, chromosome condensation in the cells continued, and the chromosomes fused into chromatin aggregates (fig. 5). Subsequently, nuclear membrane reformed around the chromatin aggregates. The cells appeared interphase-like when the

chromatin material in the nuclei became dispersed (fig. 5). Some of the interphase-like cells contained one nucleus each; others were multi-nucleated with two or more, sometimes over thirty, nuclei per cell.

Conversion of the metaphase cells to interphase-like continued as the cells were incubated at 39°C for longer periods. The fraction of interphase-like cells increased while the percent of mitotic cells diminished. The cells in interphase-like states reached a maximum of 83% at 18 h (fig. 4). Similar results were obtained when, instead of collecting cells which floated off during the 4–6 h interval, other time intervals were utilized. Transformation of the mitotic cells to mono- and multi-nucleated, interphase-like states was not due to the mitotic cells completing mitosis and then becoming interphase and multi-nucleated cells. Few, if any, cells in mitosis at 39°C were ever seen to be able to complete mitosis.

The interphase-like cells were also tested for physiological viability with trypan blue. Over 90% of the cells excluded the dye.

The above experiments show that mitotic cells, unable to complete normal mitosis, converted into interphase-like figures. Another experiment was performed to show that mutant cells, which were in interphase and had not previously entered mitosis at 39°C, could still enter mitosis at 39°C. In the experiment, cells in flasks were shifted to 39°C. At intervals after the shift, a flask was agitated and the medium was removed to eliminate the loosely attached cells. Fresh medium was added to the flask followed by a further period of incubation at 39°C. The agitation and medium removal process was repeated with the same flask. This time, however, the cells in the medium were collected by centrifugation, fixed, and stained. The same flask from which the medium was removed received fresh medium once more. After the cells were incubated at 39°C for another period of time, the agitation, fixation, and addition of fresh medium steps were again repeated. Several further rounds of the same treatment were performed with the flask.

Each time, starting from the first agitation and medium change procedure, pre-existing floating cells were removed. The subsequent agitation, cell collection, and fixation step resulted in a slide containing only the cells which floated into medium or became loosely attached during the interval between the two agitations.

If ts-546 cells were not blocked in mitosis but rather interphase cells became rounded and floated into the medium, the newly arisen floating cells should be interphase cells. On the other hand, should the ts-546 cells, which were originally attached to the flask surface in interphase, be capable of continually entering mitosis, the floating cells found between the two agitations should be mitotic.

The results showed that the floating cells collected, even after the flask had been at 39°C for 12–16 h, contained a high proportion of mitotic cells (the appearance of the cells was similar to those in fig. 5*a*). Consequently, interphase cells, which had never entered mitosis at 39°C, were capable of initiating mitosis at that temperature.

DISCUSSION

Our results showed that, after the mutant ts-546 cells grown at 33°C were shifted to the non-permissive temperature of 39°C, interphase cells continued their progress through the cell cycle until mitosis was initiated. The cells changed from a fibroblast morphology to rounded in shape and became loosely attached or not attached to

the surface of the growth chamber. Chromatin material in the cells condensed into individual metaphase chromosomes, but the subsequent mitotic events and cell division were not completed. The chromosomes fused into aggregates with nuclear membrane re-formed around the aggregates resulting in interphase-like cells containing one or more nuclei.

The above results are somewhat similar to those reported by Stubblefield [3], who demonstrated that, when cells were treated with colchicine, interphase cells continued to enter mitosis, but the mitotic cells were arrested in metaphase. Subsequently, the metaphase-arrested cells re-formed nuclear membrane and became mono- or multinucleated interphase-like cells.

The results reveal defective mitosis as an observable phenotypic expression in ts-546 cells. The defect could be the result of denaturation of a thermolabile protein essential for the completion of mitosis. An example of such a protein is tubulin or one of the proteins associated with tubulin in microtubules, or one of the other proteins in the mitotic apparatus.

Terasima & Tolmach [2] found that mitotic cells could be selectively removed from monolayer cultures by agitation. We have found the technique to be useful in some of our experiments. In other experiments, the cells we removed by agitation also include interphase-like cells which are the result of the formation of nuclear membrane around chromatin material which has not completed normal mitosis.

Hartwell et al. [4] took advantage of morphological changes during the yeast cell cycle to isolate temperature-sensitive cell cycle mutants. It appears that mitotic cell-rounding, which is characteristic of many mammalian cell lines capable of monolayer growth, may be used similarly to isolate temperature-sensitive mammalian cell mutants blocked in cell division.

Cell rounding, albeit a useful marker for mitosis, is not an absolute criterion for cell division. Willingham et al. [5] showed that a line of 3T3 mutant cells became rounded after the temperature was increased or decreased. The rounding process was a result of reduction in intracellular adenosine 3′,5′-cyclic-monophosphate levels. Mitotic mutants may show higher fractions of cells which are round in appearance; however, the lack of accumulation of rounded-up cells is not necessarily a sign that the cells contain no mitotic defects. The defective mitotic cells could conceivably become interphase-like and re-attach to the dish surface. Nevertheless, cell rounding is a useful morphological marker in our attempts to isolate mutants exhibiting mitotic defects. Some of our mutants, which accumulate rounded-up cells at the non-permissive temperature, have been found to be defective in mitosis (unpublished results).

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