Macromolecular Synthesis in a Set of Temperature-sensitive Mutants of Rat 3Y1 Fibroblasts under Proliferation-arresting and Proliferation-resuming Conditions[†]

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We characterized the macromolecular synthesis of 3 "short-survival" (3Y1tsA106, 3Y1tsB107, and 3Y1tsC113) and 4 "long-survival" (3Y1tsD123, 3Y1tsF121, 3Y1tsG125, and 3Y1tsH203) temperature-sensitive mutants of rat 3Y1 diploid fibroblasts under various conditions. Inhibition of RNA synthesis was the earliest event detectable in 3Y1tsB107 and 3Y1tsC113, and DNA synthesis was inhibited in all the long-survival mutants. In the long-survival mutants, levels of RNA synthesis of temperature-arrested and density-arrested states differed. Results of cycloheximide treatment of temperature-arrested cells after shifting down to 33.8°C suggest that *de novo* protein synthesis is necessary for DNA synthesis to resume and also that inhibition of protein synthesis delays initiation of DNA synthesis in 3Y1tsD123, 3Y1tsF121, and 3Y1tsG125.

Temperature-sensitive (ts) mutants of somatic cells are useful for understanding the reproduction process and its control in animal cells in culture.^{2,3)} Ohno *et al.*⁴⁾ isolated a large number of ts mutants from a diploid rat fibroblast line 3Y1,^{5,6)} assigned them to one of 4 classes based on viability and proliferating ability at 39.8°C (a nonpermissive temperature), and briefly described the overall pattern of macromolecular synthesis in these mutants, when cells proliferating at 33.8°C (a permissive temperature) were exposed to 39.8°C. Class I mutants (3 complementation groups), which lose viability quickly at 39.8°C ("short-survival" mutants), are inhibited in all of their DNA, RNA, and protein syntheses at 39.8°C. Class II mutants (2 complementation groups), which lose their colony-forming ability gradually and detach slowly from a plastic substratum, have DNA and RNA syntheses inhibited at 39.8°C. In class III and IV mutants (1 and 2 complementation groups, respectively) which survive much longer, their DNA

synthesis was predominantly inhibited at 39.8°C.

To study the role of macromolecular synthesis on the regulation of proliferation in these mutants, here, we undertook the following experiments. (1) The effect of a shift to 39.8°C on macromolecular synthesis in cells proliferating at 33.8°C was studied to determine if and when macromolecular synthesis ended. (2) When cells quiescent at a confluent cell density (density arrest) are stimulated with serum, protein and RNA syntheses increase, and then DNA synthesis and cell division begin in various cell lines.^{7~10)} We examined the effect of temperature on the stimulation of macromolecular synthesis in these ts mutants under the condition. (3) Using "long-survival" mutants belonging to classes II, III, and IV, we studied macromolecular synthesis after lowering the temperature of cells proliferation-arrested at 39.8°C (temperature arrest), and compared the arrest state with the density-arrested state. (4) The role of protein

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synthesis in the resumption of DNA synthesis in temperature-arrested cells was studied by inhibiting protein synthesis for various periods of time with cycloheximide (CHM).

MATERIALS AND METHODS

Cells and cell culture. The cell lines used were wild-type (WT) 3Y1 cells, 3Y1-B clone 1-6⁵⁾ (here, 3Y1) and the representatives of the 7 complementation groups of the ts mutants of 3Y1: 3Y1tsA106 (survival class I), 3Y1tsB107 (class I), 3Y1tsC113 (class I), 3Y1tsD123 (class II), 3Y1tsF121 (class III), 3Y1tsG125 (class IV), and 3Y1tsH203 (class IV).⁴⁾ Cells were cultured in Dulbecco-modified Eagle's medium (DEM) supplemented with 10% fetal bovine serum (FBS). Exponentially proliferating cells were obtained by inoculating 10^4 cells into 35-mm plastic dishes (Nunc, Roskilde, Denmark), and incubating at 33.8°C for 3 days. Temperature-arrested cells were obtained by shifting exponentially proliferating cells to 39.8°C and incubating them for 2 days. Density-arrested cells were prepared by inoculating $(2\sim4) \times 10^4$ cells into 35-mm dishes and incubating at 33.8°C for 7~10 days. Cells quiescent at confluence for 3 days were used in the serum stimulation experiments. The CO₂ incubator for the experiments at 39.8°C was placed in a walk-in incubator set at $37 \pm 1^\circ\text{C}$ to minimize fluctuations of temperature during manipulation. ³H-Thymidine (25 Ci/mmol), ³H-uridine (4.4 Ci/mmol), and ³H-leucine (1.25 Ci/mmol) were purchased from Amersham, and CHM from Sigma.

Conditions for labeling with ³H-compounds. (1) Cells proliferating at 33.8°C were labeled with either of the ³H-precursors (1 $\mu\text{Ci/ml}$ in all experiments) continuously at 33.8°C or at 39.8°C. (2) Density-arrested cells were stimulated by changing the medium to DEM containing 20% FBS, and labeled with either of the ³H-precursors for 4 hr at various intervals after serum stimulation. (3) Temperature-arrested cells were labeled with either of the ³H-precursors for 4 hr at various intervals after the temperature was brought down to 33.8°C. We chose continuous or long-pulse labeling to keep the incubation temperature as constant as possible by avoiding openings the door and manipulating the cultures.

Determination of radioactivity in the acid-insoluble fraction. Cells labeled with the ³H-precursors were washed 3 times with 2 ml each time of cold Ca²⁺-Mg²⁺-free phosphate-buffered saline, and lysed with 0.8 ml of 0.1% sodium dodecylsulfate for at least 30 min at room temperature. The lysate was mixed with 0.2 ml of 2.5 mg/ml bovine serum albumin and then precipitated with 2 ml of 10% trichloroacetic acid (TCA). The TCA-insoluble materials were collected by centrifugation at $3,000 \times g$ at 4°C for 15 min. After being washed with cold TCA, the pellets obtained were dissolved in 0.2 ml of 0.1 N NaOH and the

radioactivity was determined in a liquid scintillation counter using a toluene-triton X-100 scintillation cocktail.¹¹⁾ To compare the activities between cell lines, the count per culture was divided by the cell number at the beginning of the experiment.

RESULTS

Effects of upward temperature shifts on macromolecular synthesis in ts mutants proliferating at 33.8°C (Fig. 1)

Cells were labeled with either of the 3 ³H-precursors continuously and were lysed every 2 hr to determine if and when incorporation ceased at 39.8°C. In tsA106, all activities were inhibited substantially, but not completely, within 24 hr. In tsB107 and tsC113, RNA synthesis was completely inhibited within 6 hr, but protein synthesis continued for the first 10 hr at 39.8°C. In tsD123, DNA and RNA syntheses were completely inhibited at 36 hr. In tsF121, the cells ceased to proliferate within 6 hr, but DNA synthesis continued at a slightly slower rate for the first 12 hr after the temperature increase. RNA synthesis was also inhibited along with the DNA synthesis. Similar results were obtained in tsG125. In tsH203, cells ceased to proliferate within 8 hr and DNA synthesis was completely inhibited within 10 hr. In the long-survival mutants, the last four here, protein synthesis was not inhibited at 39.8°C.

Macromolecular synthesis at 33.8 and 39.8°C in serum-stimulated cultures of ts mutants density-arrested at 33.8°C (Fig. 2)

When density-arrested cells were stimulated with 20% serum, stimulation of the synthesis of all 3 types of macromolecules and of cell division occurred in all mutants at 33.8°C. At 39.8°C, tsD123 started DNA synthesis at 16 hr and about 50% of the cells divided at 24 hr after the stimulation, but the second round of DNA synthesis and cell division was inhibited. In tsF121 and tsG125, some leakage of DNA synthesis was observed at 39.8°C without a detectable increase in cell number. In the other mutants, DNA synthesis was inhibited completely.

FIG. 1. Macromolecular Synthesis in WT 3Y1 and ts Mutants of 3Y1 after a Temperature Shift to 39.8°C. Cells exponentially proliferating at 33.8°C were brought to 39.8°C and labeled with ^3H -thymidine, ^3H -uridine, or ^3H -leucine continuously. At intervals, cells were lysed to determine the radioactivity incorporated into the acid-insoluble fraction. (○), 33.8°C, (●), 39.8°C. Three independent experiments gave similar results.

FIG. 2. Rate of Macromolecular Synthesis in WT 3Y1 and ts Mutants of 3Y1 after Serum Stimulation. Cells density-arrested at 33.8°C were refed DEM containing 20% FBS and incubated at 33.8 or 39.8°C. Then ^3H -thymidine, ^3H -uridine, or ^3H -leucine was added at the times indicated and the preparations were incubated for 4 hr before incorporation into the acid-insoluble fraction was measured. (○), 33.8°C, (●), 39.8°C.

Induction of RNA synthesis was not observed in the short-survival mutants. In the long-survival mutants, the rate of RNA synthesis increased at 39.8°C as at 33.8°C for the first 20 hr, but thereafter ceased to increase (tsF121 and tsH203) or decreased (tsD123 and tsG125).

Protein synthesis was inhibited immediately after the temperature increase, as was RNA synthesis, in tsA106. The activity increased for the first 12 hr but decreased thereafter in

tsB107. TsC113 behaved much the same way as tsB107. In the long-survival mutants, protein synthesis at 39.8°C was as high as that at 33.8°C for tsD123 or higher than those at 33.8°C for tsF121, tsG125, and tsH203, at least for the first 24 hr.

Effect of decreased temperature on macromolecular synthesis in long-survival mutants arrested at 39.8°C (Fig. 3)

After the temperature of temperature-

FIG. 3. Rate of Macromolecular Synthesis after Shifting down to 33.8°C in Long-survival *ts* Mutants Arrested at 39.8°C.

Ts mutants arrested at 39.8°C for 2 days were brought to 33.8°C and labeled at intervals with ³H-thymidine, ³H-uridine, or ³H-leucine for 4 hr to determine the incorporation of radioactive precursors into the acid-insoluble fraction.

arrested cells was decreased, DNA synthesis occurred after a time lag of 16 hr (tsD123 and tsF121), 20 hr (tsG125), or 24 hr (tsH203). The rate of RNA synthesis increased transiently at 8 hr (tsD123 and tsG125) or at 12 hr (tsH203), and increased again in the S phase. The rates of RNA synthesis in the temperature-arrested cells (50~150 cpm/10³ cells/4 hr) were much higher than those of the density-arrested cells of the same mutants (7~11 cpm/10³ cells/4 hr), and induction of RNA synthesis was not so prominent in cells released from temperature arrest as in cells released from density arrest. Changes in protein synthesis were marked only in tsG125, with peaks in the G1 and S phases.

Effect of inhibition of protein synthesis after release from temperature arrest on initiation of DNA synthesis (Fig. 4)

To examine the possible role of protein synthesis in the initiation of DNA synthesis after release from temperature arrest, cells were treated with CHM after a temperature shift to 33.8°C for periods corresponding either to the whole time lag for DNA synthesis (16 hr in tsD123, tsF121, and tsG125, and 24 hr in tsH203) or to the first half of the lag period.

FIG. 4. Effect of CHM on the Initiation of DNA Synthesis after a Shift Down to 33.8°C in Long-survival *ts* Mutants That Had Been Arrested at 39.8°C.

Temperature-arrested cells were brought to 33.8°C. The first groups of cultures had ³H-thymidine added immediately after the shift down for a continuous labeling (○). The second groups of cultures were treated with CHM (3.3 μg/ml) for 8 hr in tsD123, tsF121, and tsG125 or for 12 hr in tsH203, and then labeled with ³H-thymidine continuously (●). The third groups of cultures were treated with CHM for 16 hr (tsD123, tsF121, and tsG125) or 24 hr (tsH203) before labeling with ³H-thymidine (▲). The fourth groups of cultures were incubated first without CHM for 8 hr (tsD123, tsF121, and tsG125) or 12 hr (tsH203), treated with CHM for 8 hr (tsD123, tsF121, and tsG125) or 12 hr (tsH203), and then labeled with ³H-thymidine (△). Two independent experiments gave similar results.

Whether treatment was for the whole period or half, initiation of DNA synthesis was delayed for either to the length of the CHM treatment period (tsH203) or longer (the other mutants; we call this "additional delay"). The additional delay after a 16-hr treatment with CHM was about 3 hr in tsF121 and 6 hr in tsD123 and tsG125. When cells were treated with CHM in the second half of the time lag for DNA synthesis, the additional delay in these 3 mutants (8~12 hr) were greater than that (2~6 hr) in cells treated for the same length of time in the first half. In tsH203, the additional delay was negligible.

DISCUSSION

We reported previously that DNA, RNA, and protein syntheses were all inhibited in short-survival mutants exposed to 39.8°C.⁴⁾ Results obtained here show that inhibition of RNA synthesis is the first detectable event in tsB107 and tsC113. It must be recalled that tsB107 of 3Y1 and the ts13 mutant of hamster BHK cells are assigned to the same complementation group.⁴⁾ In BHKts13 cells, both RNA and protein synthesis are inhibited at 39°C, but the inhibition of RNA synthesis is more marked.¹²⁾ These results suggest that RNA synthesis is first to be inhibited in both tsB107 and BHKts13 at nonpermissive temperatures.

In long-survival mutants, protein synthesis was not inhibited at all, but RNA synthesis was inhibited along with DNA synthesis in tsD123, tsF121, and tsG125. Since induction of RNA synthesis occurred at 39.8°C under conditions inhibiting DNA synthesis after serum-stimulation of density-arrested cells, inhibition of RNA synthesis in tsF121 and tsG125 seems to be a secondary effect of the inhibition of DNA synthesis. Long-survival mutants are arrested at 39.8°C predominantly with a 2C DNA content,⁴⁾ and start DNA synthesis 16~24 hr after the temperature is shifted down, as was finding by autoradiography.¹³⁾ These results suggest that ts defects in these mutants are related to the initiation of DNA synthesis and that these mutants are arrested in the states resembling those of cells in the normal G1 phase.

The temperature-arrested cells did not progress toward the S phase in the presence of CHM at 33.8°C, and initiation of DNA synthesis at 33.8°C was additionally delayed after removal of CHM in tsD123, tsF121, and tsG125. The additional delay was longer in cells treated with CHM in the second half of the time lag for DNA synthesis than in those treated in the first half. These results suggest the following: (1) that *de novo* protein synthesis is necessary for the initiation of DNA synthesis after release from temperature arrest,

and (2) that the proteins in question are synthesized mainly in the first half of the lag period for DNA synthesis, and are unstable even at 33.8°C.

Elongation of the time lag for DNA synthesis has been observed in BALB/c3T3 cells treated with CHM,¹⁴⁾ and in density-arrested C3H/10T_{1/2}¹⁵⁾ and tsG125¹⁶⁾ cells with increased arrest periods. Since the delay can be observed at 33.8°C, it may be due to degradation of proliferation-regulating proteins, probably including ts proteins in these mutants.

Induction of RNA synthesis is observed in various types of cells after serum stimulation of density-arrested cells^{7~10)} and in liver cells after partial hepatectomy.^{17,18)} After serum stimulation of density-arrested cells, it was observed in all mutants at 33.8°C and in all long-survival mutants at 39.8°C as well. Temperature-arrested cells had much higher levels of RNA synthesis than density-arrested cells, and the induction of RNA synthesis seen in density-arrested cells after serum stimulation was not observed in temperature-arrested cells after the temperature was shifted down. These findings suggest that the temperature-arrested state is different from the density-arrested state in these long-survival mutants.

REFERENCES

- 1) K. Ohno and G. Kimura, *Somat. Cell Mol. Genet.*, **10**, 29 (1984).
- 2) R. Baserga, *J. Cell. Physiol.*, **95**, 377 (1978).
- 3) S. Cooper, "Cell Growth," ed. by C. Nicolini, Plenum Press, New York, N.Y., 1982, pp. 315~336.
- 4) K. Ohno, A. Okuda, M. Ohtsu and G. Kimura, *Somat. Cell Mol. Genet.*, **10**, 17 (1984).
- 5) G. Kimura, A. Itagaki and J. Summers, *Int. J. Cancer*, **15**, 694 (1975).
- 6) T. Yamashita, K. Yamamoto, K. Shiroki, G. Kimura, K. Matsui, T. Osato, H. Shimojo and K. Fujinaga, *Int. J. Cancer*, **26**, 435 (1980).
- 7) I. Lieberman, R. Abram and P. Ove, *J. Biol. Chem.*, **238**, 2141 (1963).
- 8) J. C. Mauck and H. Green, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 819 (1973).
- 9) A. B. Pardee, R. Dubrow, J. L. Hamlin and R. E. Kletzien, *Annu. Rev. Biochem.*, **47**, 715 (1978).

- 10) N. E. Budrick and L. Miller, *J. Cell. Physiol.*, **111**, 284 (1982).
 - 11) J. C. Turner, *Int. J. Appl. Radiat. Isotopes*, **19**, 557 (1968).
 - 12) A. Taravera and C. Basilico, *J. Cell. Physiol.*, **92**, 425 (1977).
 - 13) H. Zaitzu and G. Kimura, *J. Cell. Physiol.*, **119**, 82 (1984).
 - 14) J. Campisi, E. E. Mederano, G. Morrero and A. B. Pardee, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 436 (1982).
 - 15) D. Miska and H. B. Bossman, *Biochem. Biophys. Res. Commun.*, **93**, 1140 (1980).
 - 16) H. Zaitzu and G. Kimura, *J. Cell. Physiol.*, **120**, 181 (1984).
 - 17) K. Tsukada and I. Lieberman, *J. Biol. Chem.*, **239**, 1564 (1964).
 - 18) K. Tsukada and I. Lieberman, *J. Biol. Chem.*, **239**, 2952 (1964).
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