Rapid decrease in thymidine kinase activity of mouse cell temperature-sensitive mutants at a non-permissive temperature

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A rapid decrease in the incorporation of [^3H]thymidine into DNA at a non-permissive temperature was observed in two temperature-sensitive mutants that were isolated from mouse FM3A cells. This change was not due to a decrease in the rate of DNA replication, but was closely associated with a decrease in thymidine kinase activity of these cells. Experiments to test thermolability of thymidine kinase in extracts showed that there are two components of the thymidine kinase, but there was no alteration in the sensitivity of the enzyme to high temperature. Also, the decrease in enzyme activity in the temperature-sensitive mutants at the non-permissive temperature occurred much faster than expected from the half-life of the enzyme in wild-type cells, which was measured in the presence of cycloheximide. These results suggested that the enzyme was somehow rapidly inactivated, or degraded, in the cells at the non-permissive temperature.

Methods of isolation of temperature-sensitive mutants from cultured mammalian cells have been devised and many temperature-sensitive mutants were obtained (Thompson *et al.*, 1970; Basilico & Meiss, 1974). Among these mutants, temperature-sensitive processes include aminoacyl-tRNA synthetase (Thompson *et al.*, 1973), processing of rRNA precursor (Toniolo *et al.*, 1973), cell-cycle traverse (Smith & Wigglesworth, 1974; Liskay, 1974) and DNA replication (Sheinin, 1976; Tsai *et al.*, 1979; Eilen *et al.*, 1980). These temperature-sensitive mutants should be useful for study of the mechanism of various cellular processes. For example, temperature-sensitivity is the only possible type of mutation that can be expected for functions such as DNA replication.

Temperature-sensitive thymidine kinase in a temperature-sensitive mutant (Nakano *et al.*, 1978), and inhibition of formation of thymidine kinase in another temperature-sensitive mutant at non-permissive temperature (Kit & Jorgensen, 1975), have also been reported. Correlation of the thymidine kinase activity with the regulation of DNA replication has been shown in many studies (Kit, 1976; Kit *et al.*, 1965). The present paper shows that thymidine kinase activity in two of our temperature-sensitive mutant lines was lost at the non-permissive temperature, at a rate faster than expected from the half-life of the enzyme.

Materials and methods

Materials

A clone of mouse FM3A cells, derived from mouse mammary carcinoma (Nakano, 1966), was provided by Dr. H. Kodama and Dr. H. Koyama (Cancer Institute, Tokyo, Japan) and used as a wild type. Cells were grown in suspension in Eagle's minimum essential medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 5% calf serum (GIBCO) and 0.1% Bacto-Peptone (Difco) in an atmosphere of 5% CO₂ in moist air at 34°C. Cell number was determined by a Coulter Counter model D (Coulter Electronics Co., Hialeah, FL, U.S.A.). All the radioactive compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) DEAE-cellulose paper discs (DE-81) were purchased from Whatman. Glass-fibre filters were either from Toyo Roshi (GA 100) or from Whatman (GF/C). Purified agar was from Difco. Antipain and leupeptin were gifts from Dr. M. Miyaki, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.

Isolation of temperature-sensitive mutants

A simplified method for the isolation of temperature-sensitive mutants as described by Thompson *et al.* (1970) was used. Wild-type cells were cultivated at the non-permissive temperature, 39.5°C, for

several weeks and cooled to 34°C a few days before mutagenization. The cells were treated with 0.5–1.0 µg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma)/ml for 4–6 h at 34°C. After 1 week of cell culture for the expression of mutations, cells were warmed to 39.5°C. After 8–16 h, [³H]thymidine (2 µCi/ml, 5 Ci/mmol) was added to the medium to kill selectively cells which continued to grow at this temperature. After 24–28 h of incubation at 39.5°C, cells were washed once with the culture medium and seeded on soft agar plates which contained 0.5% agar in the culture medium supplemented with 10% foetal-calf serum (GIBCO). The plates were incubated at 34°C for 2 weeks. Colonies formed were picked up, and cell growth was tested at both 34°C and 39.5°C. By this method, approx. 1–5% of the cells that survived the selection showed actual temperature-sensitive growth. We have isolated 50–60 temperature-sensitive mutant clones. In the present paper, we show the results on two clones, ts-18y and ts-31a. The character of these mutants has been stable for more than 1 year.

Uptake and incorporation of radioactive precursors

Cells in the exponential growth phase (4×10^5 – 8×10^5 cells/ml) were used in all experiments. In measuring both the uptake of [³H]thymidine into the acid-soluble fraction and its incorporation into cell DNA, 1 ml of cell culture was labelled with 1 µCi of [³H]thymidine (5 Ci/mmol)/ml for 30 min at 39.5°C. Cells were chilled in an ice bath, collected by centrifugation at 2000 rev./min for 5 min and washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954). Then 1 ml of ice-cold 4% (v/v) HClO₄ was added to the cell pellet, and the acid-soluble fraction was extracted at 0°C for 30 min, separated by centrifugation at 2000 rev./min for 10 min, and acid-soluble fraction was neutralized with 25% (w/v) KOH. Precipitates formed were removed by centrifugation as described above, and then the radioactivity was determined in a toluene scintillation mixture (Hyodo & Suzuki, 1978) containing 30% (v/v) Triton X-100.

The acid-insoluble fraction was collected on a glass-fibre filter and washed with 5% (w/v) trichloroacetic acid. The filter was then washed with ethanol, dried and the radioactivity was determined in the above toluene scintillation mixture.

For measuring the incorporation of [³H]uridine (28 Ci/mmol) or [³H]amino acids mixture (1.4–60 Ci/mmol) into the cellular macromolecules, 1 ml of cell culture was labelled with 1 µCi of radioactive precursor/ml. The labelled cells were collected by centrifugation and precipitated by ice-cold 5% trichloroacetic acid. The precipitates were collected on glass-fibre filters, washed with 5% trichloroacetic acid, and the radioactivity was determined.

Permeable-cell system for DNA replication in vitro

Exponential-phase cells (4×10^5 – 8×10^5 /ml) were exposed to 39.5°C for 0–24 h and permeabilized with 0.02% Triton X-100 as described by Hyodo & Suzuki (1978). The permeabilized cells were suspended in iso-osmotic sucrose solution (0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.8, 4 mM-MgCl₂, 1 mM-EDTA and 6 mM-2-mercaptoethanol), and a portion of the suspension was used for the assay of [³H]dTMP incorporation. Conditions for the incorporation of [³H]dTMP into cell DNA were as described by Hyodo & Suzuki (1978).

Assay of thymidine kinase activity

Approx. 4×10^7 cells were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline. Then cells were suspended to 0.5 ml of extraction buffer (50 mM-Tris/HCl, pH 7.5, 1 mM-2-mercaptoethanol, 1 mM-MgCl₂, 0.1 M-KCl and 0.5 mM-phenylmethanesulphonyl fluoride) and were disrupted by sonication with four 20 s pulses at power 3 of a Branson Sonifier with a microtip. The lysate was centrifuged at 10000 g for 30 min. A portion of the supernatant fraction was used for the assay of enzyme activity.

The reaction mixture (0.1 ml) for the assay of thymidine kinase activity contained 0.2 M-Tris/HCl, pH 7.5, 3 mM-MgCl₂, 3 mM-ATP, 10 mM-2-mercaptoethanol, 10 mM-NaF, 0.2 mM-[³H]thymidine (10 mCi/mmol) and 20 µl of cell-free extract. Cell extract concentration in the reaction mixture (0.8 mg of protein/ml) was equivalent to 1.5×10^7 cells/ml. After 30 min of incubation at 37°C, the reaction was stopped by chilling the mixture in an ice bath, and a 50 µl sample was applied to a DEAE-cellulose paper disc (Whatman DE-81). Paper discs were batch washed with four changes of cold 1 mM-ammonium formate and once with ethanol. The discs were dried, and the radioactivity binding to them was determined in a toluene scintillation mixture (Hyodo & Suzuki, 1978). Thymidine kinase activity was calculated as pmol of TMP formed/30 min per 10⁶ cells or per mg of protein and expressed as a percentage of the control (cells grown at 34°C). The results were essentially identical in both cases, whether the enzyme activity was expressed per number of cells or per mg of protein. Protein concentration was determined by the method of Lowry *et al.* (1951).

Results

Temperature-sensitive mutants isolated from mouse FM3A cells

Figs. 1(a) and 1(b) show the growth curves of ts-18y and ts-31a cells respectively. The doubling time of the wild-type cells was 16 h at 34°C, 11.5 h at 37°C and 15 h at 39.5°C, and their saturation

Fig. 1. Growth of *ts-187* (a) and *ts-31a* (b) cells

Cell number was determined with a Coulter Counter. Cells were incubated at 34°C (O) or at 39.5°C (●).

density under our culture conditions was 2×10^6 – 2.5×10^6 cells/ml at each temperature. The *ts-18y* cells show a longer doubling time than that of the wild-type cells, 24 h at 34°C, and a lower saturation density, approx. 1×10^6 /ml. The *ts-31a* cells had a doubling time and saturation density similar to those of the wild-type cells at 34°C. When shifted to 39.5°C, *ts-18y* cells doubled in cell number before they stopped growing, whereas *ts-31a* cells increased by only 10%. When these cells were kept at 39.5°C, the DNA content analysed by a flow-cytometric method (Krishan, 1975) showed no specific arrest in the cell cycle.

Uptake and incorporation of radioactive precursors by temperature-sensitive mutants

The results are shown in Figs. 2(a) (*ts-18y* cells) and 2(b) (*ts-31a* cells) for the incorporation of radioactive precursors into cellular macromolecules. In both mutants, rates of incorporation of [3 H]uridine and [3 H]amino acids increased after the temperature shift and reached values of 200–300% of the controls (cells grown at 34°C). Similar increases in incorporation of these precursors were observed also in the wild-type cells. In contrast, a rapid decrease was observed for the incorporation of [3 H]thymidine in these mutants at 39.5°C (Fig. 2). These results may suggest that DNA replication in these cells was affected at 39.5°C. We next examined the DNA replication in these cells, using the permeabilized cell system. In this system, the intactness of the DNA replication machinery is preserved, and continuation of the DNA replication *in vivo* can be measured with no artefacts such as repair-type synthesis (Hyodo & Suzuki, 1980). The rate of DNA replication in the permeabilized cells was calculated to be 70–80% of that of the intact cells (Hyodo & Suzuki, 1978).

Both of the temperature-sensitive mutants were

cultivated at 39.5°C for 0–24 h and then assayed for the incorporation of [3 H]dTMP into DNA (Figs. 2a and 2b). Unlike the decreased incorporation of [3 H]thymidine into intact cells, both *ts-18y* and *ts-31a* cells showed no rapid decrease in the incorporation of [3 H]dTMP into DNA. The ability of these cells to continue DNA replication was not greatly affected at 39.5°C. Thus the temperature-sensitive growth of these mutants was not due to the direct inhibition of DNA replication at the non-permissive temperature. There could therefore be a temperature-sensitive step in either uptake or phosphorylation of thymidine by these cells. We then measured the uptake of [3 H]thymidine into the cellular acid-soluble pool. The results (Figs. 2a and 2b) show that the rate of uptake of [3 H]thymidine into the cellular pool was decreased rapidly, with the same rate as the decrease in the incorporation of [3 H]thymidine into the acid-insoluble fraction. The results support the possibility that there is a temperature-sensitive defect in thymidine metabolism.

Thymidine kinase activity of the FM3A cells

We then tested the phosphorylation process of thymidine in both of the temperature-sensitive mutant cells. Thymidine kinase activity, which is involved in the first step of the phosphorylation of thymidine, was examined after the temperature shift. The results (Fig. 3) show that thymidine kinase activity decreased rapidly after the temperature shift and at the same rate as the decrease in the uptake or incorporation of [3 H]thymidine. Thus the decrease in the uptake of thymidine may be closely related to the decrease in thymidine kinase activity.

There are at least two possible changes in these mutants which would account for the decrease in thymidine kinase activity. One is the mutational alteration of the enzyme molecule, which would produce a thermolabile enzyme. Another possibility

Fig. 2. Uptake and incorporation of the radioactive precursors by *ts-18y* (a) and *ts-31a* (b) cells

Cells were incubated at 39.5°C for 0–24 h. Then cells were labelled with [^3H]thymidine ($\square$, acid-soluble fraction; Δ , acid-insoluble fraction), [^3H]uridine ($\circ$) or [^3H]amino acids mixture ($\blacktriangle$) (each $1\mu\text{Ci/ml}$) for 30 min at 39.5°C. Radioactivity in the acid-soluble or acid-insoluble fraction was determined as described in the Materials and methods section. Also, after incubation of the cells at 39.5°C, cells were permeabilized and labelled with [^3H]TTP for 30 min at 39.5°C ($\bullet$) by methods described previously (Hyodo & Suzuki, 1978). Incorporated radioactivity (c.p.m./ 10^6 cells) was expressed relative to that for the cells incubated at 34°C (100%).

is the rapid inactivation, or degradation, of the enzyme in the cells. To test the first hypothesis, we determined the effect of increasing temperature on the enzyme activity of the postmicrosomal fraction.

Cell-free extracts were obtained from the wild type and mutants grown at 34°C. Then the extracts were incubated at 0–85°C for 3 min before the assay of thymidine kinase activity. The results (Fig. 4) show that both temperature-sensitive mutants had lower enzyme activity per cell than did the wild-type cells. It was also evident that the activity has two components, differing in thermolability. The first component was inactivated during the preincubation at 25–40°C, and the second component at 55–70°C. The first component consists of 10–15% of the total activity of the wild-type cells, and both mutants possessed approximately the same amount of this component per cell. The amount of the second component varied among the clones, which resulted in the different total enzyme activities. There was no difference between wild-type and temperature-sensitive mutants in the temperature at which the inactivation of the enzyme activity of each component occurred. These results show that the decrease in enzyme activity in temperature-sensitive mutants at the non-permissive temperature was not

likely to be due to the mutational alteration of the enzyme, but it could be due to an inactivation, or degradation, of the enzyme in the cells.

We then determined the half-life of thymidine kinase activity in the wild-type and mutants at different temperatures in the presence of cycloheximide. Cycloheximide ($10\mu\text{g/ml}$) was added to the culture of wild-type cells at 34°C or at 39.5°C, and to temperature-sensitive mutants at 34°C. After incubation of the cells for 0–9 h, enzyme extracts were prepared and the thymidine kinase activity was measured. The results (Fig. 5) indicate that in wild-type cells the half-life of enzyme activity was slightly different at 34°C and 39.5°C. From the slopes in Fig. 5(a), the half-life of the enzyme activity was estimated to be approx. 15 h at 34°C and 8 h at 39.5°C. In both of the temperature-sensitive mutants, thymidine kinase activity showed the same decay rate as the wild-type cells at 34°C (Fig. 5b). These results show that the inactivation, or degradation, of thymidine kinase activity at the non-permissive temperature in these temperature-sensitive mutants (Fig. 4) occurred much faster than expected from the half-life of the enzyme. These results support the possibility that unusually rapid inactivation of thymidine kinase activity occurred in

Fig. 3. Thymidine kinase activity in *ts-18y* (□) and *ts-31a* (△) cells

Cells were incubated at 39.5°C for 0–8 h in the culture medium. After the incubation, thymidine kinase activity in the cell-free extract was determined. The reaction mixture was incubated at 37°C for 30 min; 100% activity corresponds to 0.69 nmol/30 min per mg of protein (*ts-18y*) or 1.2 nmol/30 min per mg (*ts-31a*).

the mutants at the non-permissive temperature.

Neff *et al.* (1979) showed that the degradation of proteins with longer half-lives was inhibited by bacterial proteinase inhibitors, but that the degradation of short-lived ones was not. We therefore tested the effect of proteinase inhibitors (leupeptin and antipain) on the rate of inactivation of thymidine kinase. The results indicate that the rapid inactivation of thymidine kinase was not affected by these proteinase inhibitors (results not shown).

Discussion

In two of the temperature-sensitive mutants, *ts-18y* and *ts-31a*, isolated from mouse FM3A cells, a rapid decrease in the incorporation of [³H]-thymidine was observed. Although the rate of decrease in the incorporation of [³H]thymidine into DNA was similar (Fig. 2), these two mutants showed a difference in the cell-number increase after the temperature shift (Fig. 1). Thus we suspected that the decreased rate of incorporation of [³H]-thymidine might not reflect the rate of DNA replication in the cell. Then experiments using permeabilized cells indicated that the temperature-sensitive growth of these mutants was not due to the direct inhibition of DNA replication at the non-permissive temperature.

A rapid decrease in uptake of [³H]thymidine was then observed in these temperature-sensitive mutants after the temperature shift to 39.5°C. This decrease in these mutants could be due to a change in either the transport of thymidine across the cell membrane or its phosphorylation steps in the cells. We tested

Fig. 4. Thermolability of thymidine kinase activity Wild-type (○), *ts-18y* (□) or *ts-31a* cells (△) were grown at 34°C and harvested. The extract was prepared as described in the Materials and methods section. Portions of the extracts were incubated for 3 min at the temperatures indicated. The extracts were then added to the reaction mixture, and thymidine kinase activity was measured at 37°C for 30 min. Enzyme activity per 10⁶ cells was determined, and the activity in the extract of the wild type not preincubated was set as 100%, which corresponds to 1.7 nmol/30 min per 10⁶ cells.

Fig. 5. Decay of thymidine kinase activity in the presence of cycloheximide

(a) Wild-type cells were incubated with cycloheximide (10 µg/ml) at 34°C (○) or at 39.5°C (●) for the times indicated. Then thymidine kinase activity was determined. (b) *ts-18y* (□) or *ts-31a* cells (△) were incubated with cycloheximide (10 µg/ml) at 34°C for the time indicated. Then thymidine kinase activity was determined.

the first phosphorylation step of thymidine in these cells, and the rapid decrease in thymidine uptake was found to be closely associated with the decrease in thymidine kinase activity. Transport of thymidine by the cell may also be affected, since the coupling of transport of nucleosides and nucleoside kinases have been suggested (Hauschka, 1973). No such decrease was observed with wild-type cells. When the extracts of wild-type and of either temperature-sensitive mutants were mixed, it was revealed that the decrease in the enzyme activity in the mutants was not due to the formation of inhibitory substance (results not shown).

There are some reports on the change in thymidine kinase activity in mammalian temperature-sensitive mutants. Nakano *et al.* (1978) reported that such a mutant isolated from FM3A cells, from a clone deficient in thymidine kinase activity (5-bromodeoxyuridine-resistant cells), possessed thermolabile thymidine kinase. Kit & Jorgensen (1975) showed that formation of thymidine kinase and deoxycytidylate deaminase were inhibited in a temperature-sensitive mutant isolated from Chinese-hamster cells. They found that, after the addition of medium with 10% serum to serum-depleted cultures, cytosol thymidine kinase increased when the cultures were incubated at 36.5°C, but not at 40.5°C, where the initiation of DNA replication was affected.

Differing from these reports, our results suggest that rapid inactivation, or degradation, of thymidine kinase activity occurred after the temperature shift, and the rate of decay of the enzyme activity was much faster than expected from the half-life of the enzyme in wild-type cells measured at 39.5°C. The results may be related to the report by Bouche *et al.* (1979), which showed that after 1 h of incubation of Chinese-hamster ovary cells at 43°C, changes in protein distribution and synthesis occurred. They showed that a 45 000-dalton protein in cytoplasm disappeared rapidly, while some other proteins were induced. Although the origin of the cells and the temperature used were different, a similar mechanism might have worked in our temperature-sensitive mutants. A degradative process might be activated at the non-permissive temperature and cause the inactivation of thymidine kinase or possibly some other proteins necessary for cell growth. If this happened, then the loss of thymidine kinase and cell growth at 39.5°C may be connected.

Our study on the thermolability of thymidine kinase revealed that the extracts of both wild-type and mutants contained two components that were inactivated at different temperatures. The results (Fig. 4) showed that the ratio of these two components in extracts were different among these

cell clones, suggesting a differential expression of the genes. The origin of this heterogeneity is not known. Since disc polyacrylamide-gel electrophoresis of extracts from wild-type and temperature-sensitive cells showed a single slow-migrating peak of the enzyme activity (results not shown), it was probably not due to the presence of mitochondrial thymidine kinase in addition to the cytosol enzyme. The differential expression of the components among these cell clones seems to suggest the existence of isoenzymes (Ohashi & Taguchi, 1976).

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